Caffeine as a probe substance to study genetic polymorphism

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CAFFEINE AS A PROBE SUBSTANCE TO STUDY GENETIC POLYMORPHISM

Submitted by

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"There appears to be no reason why cancer attacks one individual and spares others exposed to the same external conditions. It is this failure to account for the apparently arbritary selection of its victims which gives to the disease the air of a grim mystery.

The explanation of this failure is to be found in the fact that up to now such comparisons have been made by taking into account only the extrinsic factor as the determining condition and neglecting altogether the complicating influence of the intrinsic factor of susceptibility."

Cramer. W. (1934).

Dedication

To my parents and Chris

for all their support and encouragement

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ABBREVIATIONS

y	- year
h	- hour
min	- minute
sec	- second
mg	- milligram
ml	- millilitre
u	- microlitre
mm	- millimetre
MR	- Metabolite ratio
RT	- Room temperature
FAP	- Familial adenomatous polyposis
OCS	- Oral contraceptive steroids
HRT	- Hormone replacement therapy
BMI	- Body Mass Index
CBT	- Caffeine breath test
BP	- Benzo[a]pyrene
AHH	- Arylhydrocarbon hydroxylase
PAH	- Polycyclic aromatic hydrocarbons
P450	- Cytochrome P450
NAT	- N-Acetyltransferase
3-MC	- 3-Methylcholanthrene
1-MX	- 1-Methylxanthine
1-MU	- 1-Methyluric acid
1,7-DMX	- 1,7-Dimethylxanthine (paraxanthine)
1,7-DMU	- 1,7-Dimethyluric acid
1,3-DMX	- 1,3-Dimethylxanthine (theophylline)
3,7-DMX	- 3,7-Dimethylxanthine (theobromine)
AFMU	- 5-Acetylamino-6-formylamino-3-methyluracil
AAMU	- 5-Acetylamino-6-amino-3-methyluracil
DMMIOD	- 1,3-Dihydro-1-methyl 1-5-(methylamino)-8-imidazo-[4,5-d]]1,3] oxazine-2,7-dione.

SUMMARY

Interindividual differences in the biotransformation of drugs and other xenobiotics, whether caused by genetic or environmental factors or their interaction, may have important clinical consequences in drug therapy and chemical toxicity.

The use of caffeine as an *in vivo* probe for the assessment of variability in hepatic drug metabolising enzymes in human populations was investigated. Population studies revealed a considerable degree of variation in the pattern of urinary metabolites excreted, following consumption of caffeine containing beverages. Caffeine metabolite ratios were found to be reproducible and unaffected by either the time of urine collection or the amount of caffeine consumed. A simple caffeine test was thus developed, involving collection of a spot urine sample 2-6 h after caffeine consumption, for widespread use and measurement of P450IA, NAT and xanthine oxidase activities.

The metabolite ratio of (AAMU + 1-MU + 1-MX)/1,7-DMX was used to determine P450IA activity. The frequency distribution for P450IA activity appeared to be bimodal with 9% of the control population exhibiting high activity. The degree of enzyme activity was significantly altered by cigarette smoking and a greater proportion of smokers had high P450IA activity than nonsmokers. The proportion of patients with carcinoma of the lung, head and neck with high P450IA activity was significantly greater than that of controls. The proportion of leukaemia, colorectal cancer and FAP patients with high P450IA activity was not significantly different from controls.

The metabolite ratio used to determine P450IA2 activity was

(AAMU + 1-MU + 1-MX + 1,7-DMX)/Caffeine. The frequency distribution for this enzyme activity also appeared to be bimodal. 16.5% of the control population exhibited high P450IA2 activity and significantly more smokers had high activity compared to nonsmokers. The proportion of FAP patients with high P450IA2 activity was significantly greater than that of controls. The proportion of patients with colorectal cancer, lung, head and neck cancer and leukaemia who had high P450IA2 activity however, was not different to that of controls.

The metabolite ratio of (AAMU + 1-MU + 1-MX)/AAMU was used to determine NAT activity. The frequency distribution was bimodal and 59% of control volunteers were slow acetylators. NAT activity was significantly altered by age. A greater proportion of subjects over 60 y of age were slow acetylators, as others have found. There was a significantly greater percentage of slow acetylators in patients with FAP and their first degree relatives, compared to age-matched controls. The proportion of slow acetylators in patients with neoplastic disorders however, was not altered compared to control volunteers.

Preliminary studies using cDNA expressed cytochrome P450IA1 suggested that this isozyme of the P450IA family was capable of metabolising both caffeine and 1,7-DMX. Should P450IA1 be induced in human liver, it may be involved in caffeine metabolism, in addition to P450IA2.

CHAPTER ONE

INTRODUCTION

1.1 DRUG METABOLISM

Most of the xenobiotics to which the human body is exposed are lipid soluble, weak electrolytes which are readily absorbed across membranes and distributed throughout the body. After filtration by the glomerulus, these compounds are likely to be reabsorbed by the renal tubules unless they become chemically modified to form more polar derivatives. Metabolism of drugs results in the production of molecules which are usually less lipid soluble, more strongly ionised than those from which they are derived and therefore excreted more quickly. Consequently, excretion of xenobiotics is often preceded by their biotransformation into more polar metabolites.

The enzyme systems responsible for such metabolism are traditionally divided into two groups; Phase I and II. During Phase I metabolism, one or more water soluble groups are attached to the lipid soluble parent molecule, such that a position is formed for Phase II or "conjugating" enzymes to attack. Many Phase I, and especially the Phase II metabolites are sufficiently water soluble to be excreted from the body (Curry, 1977).

1.2 CYTOCHROMES P450

The cytochrome P450 monooxygenase system is a family of enzymes (Conney, 1980; Nebert & Negishi, 1982; Nebert *et al*, 1989; Puga & Nebert, 1990) which have different, but overlapping substrate specificities for a diverse group of substrates, in a wide

variety of tissues (Yang & Lu, 1987).

"Cytochrome", derived from the Greek means "coloured substance in the cell" and "P450" denotes the reddish pigment of CO-binding haemoprotein that has its major optical absorption peak at 450nm. The ubiquitous cytochromes P450 catalyse the majority of Phase I oxidations and metabolise a vast array of chemicals and endogenous substances (Nebert, 1979).

In order to oxidise such chemicals, the P450 haemoproteins receive electrons from the cofactors NADPH and/or NADH. These electrons are received one at a time, usually via reductases, which are flavoproteins. The reduced drug-P450 complex then combines with oxygen to form an active complex, from which oxidised drug and water are released. This electron chain is located principally in the ER, to some degree in the inner mitochondrial membrane and perhaps the nuclear envelope (Nebert et al, 1980a,b).

The cytochrome P450 system is primarily concerned with xenobiotic detoxification but can activate several different classes of chemicals into reactive metabolites, which may be involved in the pathogenesis of cytotoxicity (Gillette, 1980; Nelson & Harvison, 1987; Nebert et al, 1987; Guengerich, 1988). The enzymes involved in detoxification and toxication coexist in the same cells, are likely to differ among various tissues and are controlled by many host factors, such that a delicate balance exists between these two processes (Nebert, 1979).

1.3 CYTOCHROME P450IA

The P450IA family has two genes, CYPIA1 and CYPIA2 on chromosome 15 (Wolf *et al*, 1990) which encode P450IA1 and P450IA2 (Hildebrand *et al*, 1985; Jaiswal *et al*, 1987). The IA1 and IA2 genes are ubiquitous in mammals and for the most part, have similar catalytic activity in several species. Cytochromes P450IA appear to be involved in the conversion of relatively inocuous chemicals to reactive electrophilic metabolites which may react covalently with cellular macromolecules (Miller & Miller, 1981), rather than detoxification. Other P450 cytochromes tend to direct metabolism towards the formation of inactive metabolites and detoxification (Creaven & Parke, 1966; Ioannides & Parke, 1987). Although P450IA1 and IA2 show overlapping substrate specificity, P450IA1 tends actively to metabolise PAH, which are ubiquitous products of combustion, into carcinogenic metabolites (Nebert, 1981b; Kadlubar & Hammons, 1987). P450IA2 exhibits a high level of catalytic activity towards arylamine compounds, in addition to metabolising potent promutagens derived from pyrolysates of proteins (Thorgeirsson & Nebert, 1977; Kamataki *et al*, 1983; Hammons *et al*, 1985; Shimada & Okuda, 1988; Aoyama *et al*, 1989; Butler *et al*, 1989a; Shimada *et al* 1989; Batula *et al*, 1990; Gonzalez *et al*, 1990) (Fig.1.1).

Most chemical carcinogens, except direct alkylating agents, require metabolic activation *in vivo* to electrophiles, before they become ultimate carcinogens. Such chemicals seek to react with nucleophilic sites, which are abundant in DNA, RNA and other proteins. As some chemicals are activated to several carcinogenic products, and there are several nucleophilic sites

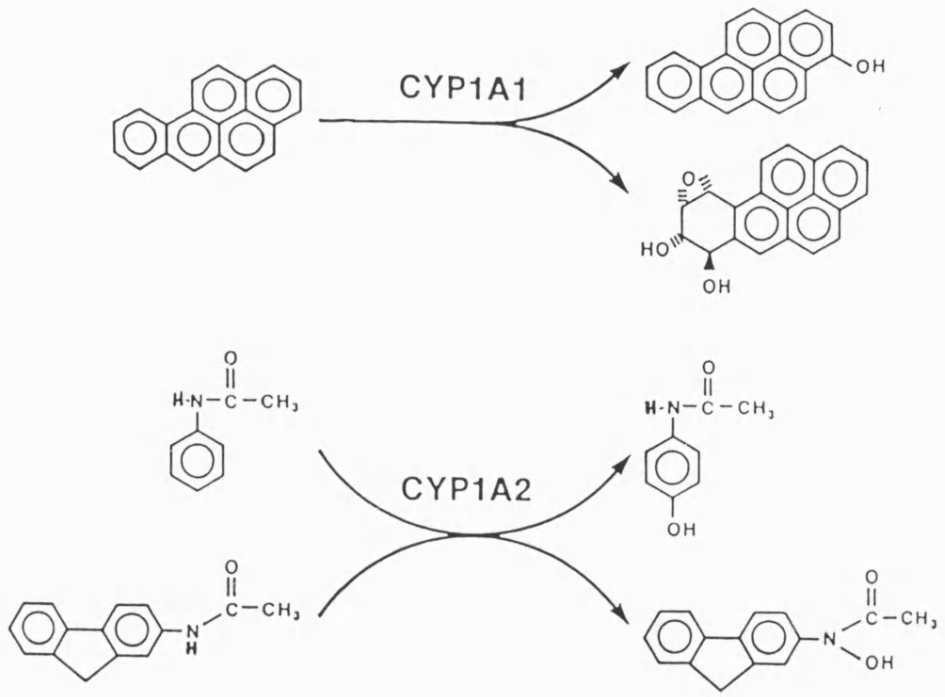


Figure 1.1 - Hydroxylation of BP by P450IA1 and arylamines by P450IA2 (Nebert, 1991).

in each macromolecule, multiple adducts are possible and frequently observed (Miller, 1978; Miller & Miller, 1981). Chemical carcinogenesis has long been associated with the induction of P450IA (Creaven & Parke, 1966) and it has been suggested that this family of enzymes is specifically concerned with the activation of chemicals to reactive intermediates and the formation of mutagens and carcinogens (Phillipson et al, 1982; Ioannides et al, 1984; Lewis et al, 1986; Ioannides & Parke, 1987).

Both P450IA enzymes could therefore play potentially important roles in chemically induced human cancer (Gonzalez, 1989).

Individual variation in cytochrome P450IA activity would give rise to differences in drug metabolism and pharmacological response (Wolf et al, 1990). The extent to which variability in

CYPIA expression can be attributed to genetic factors in man is unknown but environmental factors are important, as genes within the CYPIA family are inducible with drugs and environmental agents (Conney, 1967).

Recent studies have shown that the degree of induction of P450IA1 correlates well with the carcinogenicity of PAH chemicals (Ayrton et al, 1990a) and that P450IA2 induction by arylamines also correlates with the carcinogenicity or mutagenicity of these chemicals (Ayrton et al, 1990b). Thus chemical carcinogens not only induce the P450IA enzymes but are preferentially metabolised by these enzymes (Ioannides & Parke, 1990).

Location

P450IA1 is found in many tissues following inducer treatment (Gonzalez, 1989), while P450IA2 is only measured in the liver. In general, the form of P450 analogous to P450IA2 predominates over that of P450IA1 in rat (Goldstein & Linko, 1983) and mouse liver (Negishi & Nebert, 1979) and a similar situation appears to occur in man (Wrighton et al, 1986; Jaiswal et al, 1987; Sesardic et al, 1988). While some studies have failed to measure P450IA1 in human liver (Sesardic et al, 1988), several others have found it to be present in small quantities, particularly in heavy smokers (Quattrochi et al, 1985; Wrighton et al, 1986; Jaiswal et al, 1987; Cresteil & Eisen, 1988; Ikeya et al, 1989). P450IA1 is present in the placenta of smokers (Fujino et al, 1982; Wong et al, 1986; Pasanen et al, 1989; Vahakangas et al, 1989), in human lymphocytes (Fujino et al, 1982), breast tissue

(Vickers et al, 1989), skin (Ichikawa et al, 1989) and bone marrow (Schnier et al, 1989).

1.4 FACTORS AFFECTING DRUG METABOLISM

The process of drug biotransformation in man may be influenced by a multitude of environmental factors, such as cigarette smoke (Vahakangas et al, 1983), air pollution (Pike et al, 1975), and diet (Reddy et al, 1980; Miller, 1982), as well as host factors (Cole, 1982), including age (Greenblatt et al, 1980), race (Kalow, 1985; Miller, 1986), sex (Kato, 1974), pregnancy (Lambert et al, 1987), infectious disease (Renton & Knickle, 1990) and genetic constitution (Thorgeirsson & Nebert, 1977; Nebert, 1980a; Nebert, 1981a) that are intimately interrelated (Vesell, 1977; 1982; 1984) (Fig.1.2). Thus, attempts to separate environmental from genetic contributions as though they were discrete, unrelated entities is often invalid as many environmental factors that alter rates of drug disposition do so by affecting genetic mechanisms.

Environmental

Environmental factors play an important part in the variability of drug disposition, especially when the cytochrome P450 enzymes are involved, as their activity is markedly affected by environmental exposure to inducing and inhibiting agents (Conney, 1967; Nebert, 1980c; Conney, 1982).

Cigarette smoke contains numerous compounds which are capable of inducing certain P450 enzymes and in this way, the metabolism of phenacetin (Sesardic et al, 1988), caffeine (Campbell et al, 1987) and antipyrine (Vahakangas et al, 1983) is increased in

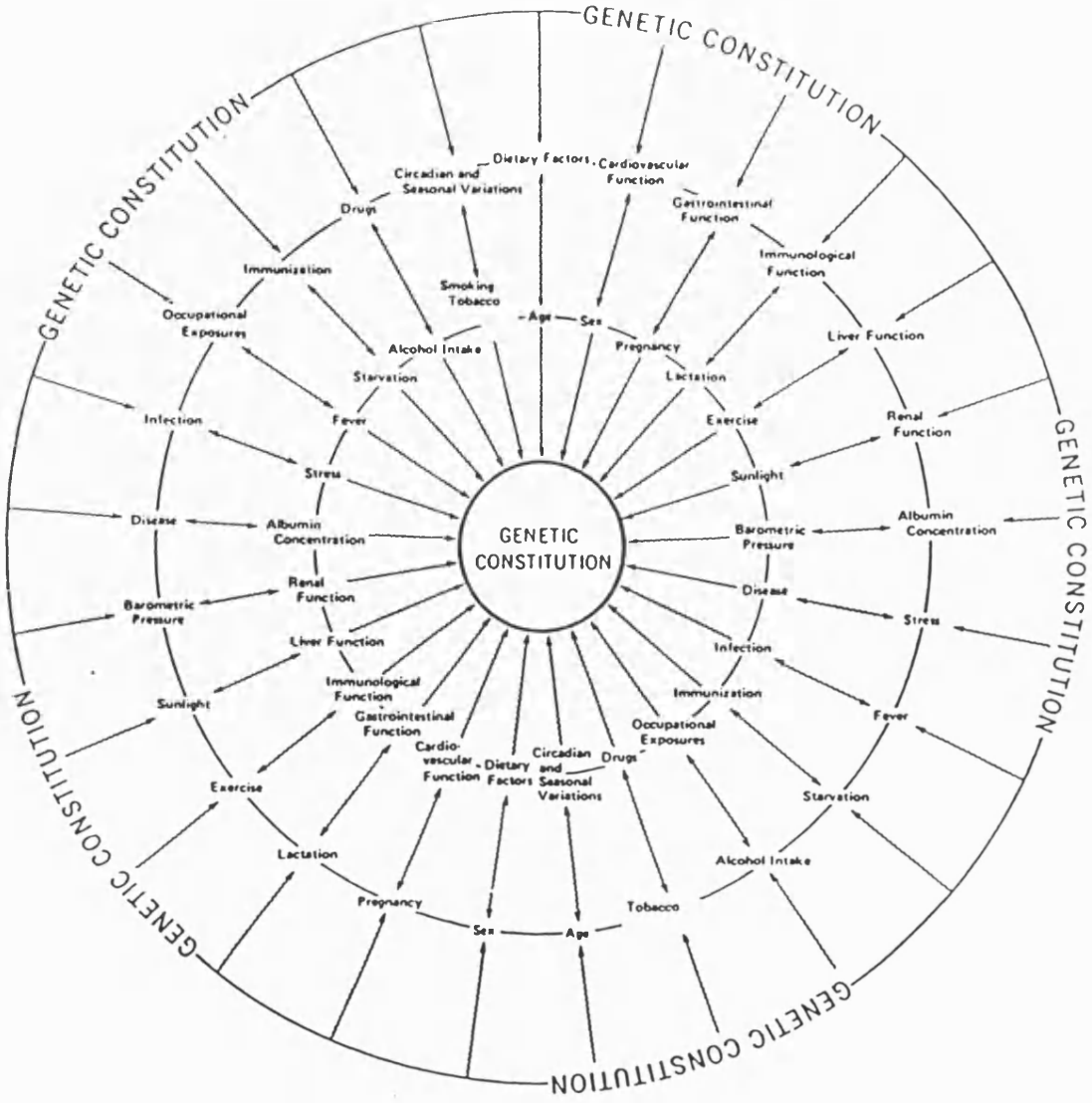


Figure 1.2 - The multiplicity of factors influencing drug response in man. The concentric outer circles emphasise the multiple possibilities that exist for interaction among host factors and suggest that the magnitude of the impact of host factors on drug response may be modulated be genetic constitution (Vesell, 1982).

smokers compared to nonsmokers.

Diet is also an important source of variation of xenobiotic metabolism (Conney, 1980). The metabolism of BP by human liver is stimulated by PAH in charcoal-broiled beef (Conney, 1980) and sensitive to changes in the content of cruciferous vegetables, such as indoles and flavones present in sprouts and cabbage (Wattenberg, 1978; Wattenberg & Loub, 1978).

Pharmacogenetics

Studies of variation in drug response due to genetic factors are the foundation of the discipline termed pharmacogenetics, which aims to assess the genetic contribution to individual differences in sensitivity to drugs and xenobiotics.

A polymorphism was described by Harris (1976) as "a type of variation in which individuals with sharply distinct qualities co-exist as normal members of a population". The molecular basis for such variation lies in the occurrence of mutant alleles at a given gene locus (or loci) encoding for an enzyme, resulting in differences in activity due to varying amounts or structures of that enzyme.

Some genetic variants are rare, occurring only in family groups while others are polymorphic, that is, the frequency of the least common allele exceeds 1% (Harris, 1976; Jackson et al, 1991). Genetic polymorphism as a phenomenen is extremely common and Harris (1976) has suggested that at least a third of human gene loci are multiallelic.

There may be monogenic or polygenic inheritance of a particular trait, for example a metabolic drug reaction, and in order to

distinguish between the frequency distributions of the investigated parameter, population studies are usually employed (Vesell, 1977; Breimer, 1983). If the frequency distribution displays bi- or trimodal characteristics, with each mode corresponding to a different genetically determined phenotype, then inheritance is monogenic and there is simple Mendelian segregation of two alleles at a single locus. However, if the frequency distribution is unimodal, inheritance is polygenic and at least two genes at different loci control the metabolic reaction (Eichelbaum, 1982).

With polygenic inheritance it is more difficult to distinguish between the genetic and environmental factors contributing to the observed variability and in order to do so family pedigree analyses and studies in mono- and dizygotic twins need to be employed (Vesell & Penno, 1983).

1.5 PHARMACOGENETIC DEFECTS IN DRUG BIOTRANSFORMATION

Among the first polymorphisms to be discovered was that controlling the disposition of the tuberculostatic drug, isoniazid (Evans et al (1960). The observed variation was found to be related to differences in the rate of acetyl coenzyme A-dependent acetylation, which is catalysed by liver cytosolic arylamine N-acetyltransferase (NAT) (Grant et al, 1990). Three human NAT genes have been assigned to chromosome 8; gene 1 encodes polymorphic NAT; gene 2 has multiple mutations and does not encode a functional NAT protein, while gene 3 encodes monomorphic NAT (Blum et al, 1990).

The first clear indication of polymorphism involving cytochrome P450 enzymes was demonstrated by Mahgoub et al (1977). CYPIID6,

the gene controlling debrisoquine hydroxylation in man is multiallelic. Three different forms of incorrectly spliced P450IID6 pre-mRNAs have been identified in the livers of the poor metabolizer phenotypes (Gonzalez, 1989) such that functionally active proteins are not expressed.

There are several other forms of polymorphism in drug oxidation which do not cosegregate with that for debrisoquine. Metabolism of the anticonvulsant, S-mephenytoin by P450IIC9 is subject to genetic polymorphism (Kupfer et al, 1984; Wedlund et al, 1984; Inaba et al, 1985; Kalow, 1986) and particularly affects Oriental subjects. The same enzyme also contributes to the metabolism of diazepam (Bertillson et al, 1989) and possibly some anticancer drugs (Relling et al, 1989). Genetic polymorphism has also been reported for the metabolism of phenacetin (Distlerath et al, 1985), the mucolytic agent S-carboxymethylcysteine (Waring, 1980; Waring et al, 1982), amobarbital (Kalow et al, 1977), theophylline (Dahlqvist et al, 1984; Miller et al, 1985; Vesell, 1986) and antipyrene (Vesell & Page, 1968; Penno & Vesell, 1983; Vesell, 1986). Metabolism of the anticonvulsant phenytoin (Vermeij, 1988) and the oral hypoglycaemic agent, tolbutamide (Scott & Poffenbarger, 1979) is also subject to genetic polymorphism, although the latter two reactions may be catalysed by the same P450 isozyme (Doecke et al, 1991).

Polymorphism of Cytochrome P450IA

In mice, the Ah locus, which controls the induction of P450IA enzymes is inherited in a Mendelian dominant fashion, such that

Ah-responsive (Ah^bAh^b) and Ah-nonresponsive (Ah^dAh^d) mice exist (Nebert et al, 1975; Thorgeirsson & Nebert, 1977; Nebert & Felton, 1975). Due to the fact that P450IA1 can convert procarcinogens to ultimate carcinogens and reactive intermediates, differences in cancer risk have been demonstrated in mice treated with PAH (Kouri, 1976; Kouri et al, 1980; Nebert, 1981b). When carcinogens such as PAH are placed in direct contact (via subcutaneous, topical or intratracheal routes) with the tissue being studied, the genetically responsive mouse is at increased risk for developing a tumour in that tissue, compared to the nonresponsive mouse that receives the same dose of chemical. This is due to increased induction of P450IA and therefore increased carcinogen activation in tissues close to the site of chemical application in the responsive mouse (Nebert, 1982).

On the other hand, PAH given orally or subcutaneously cause malignancy and toxicity in tissues distant from the site of chemical administration in the nonresponsive mouse. Following oral administration of BP, Nebert et al (1980) showed that a 20-fold higher uptake of the drug occurred in the marrow and spleen of nonresponsive, compared to responsive mice. The concentration of nonmetabolised parent drug that reaches distal tissues is much greater in the nonresponsive animals and is responsible for the increased toxicity and tumorigenesis observed in such tissues (Fig.1.3).

The responsive mouse is more prone to chemically induced pulmonary tumours, fibrocarcomas, epidermal carcinoma, fetal toxicity and cleft palate in the fetus, whereas the nonresponsive mouse is more prone to chemically induced

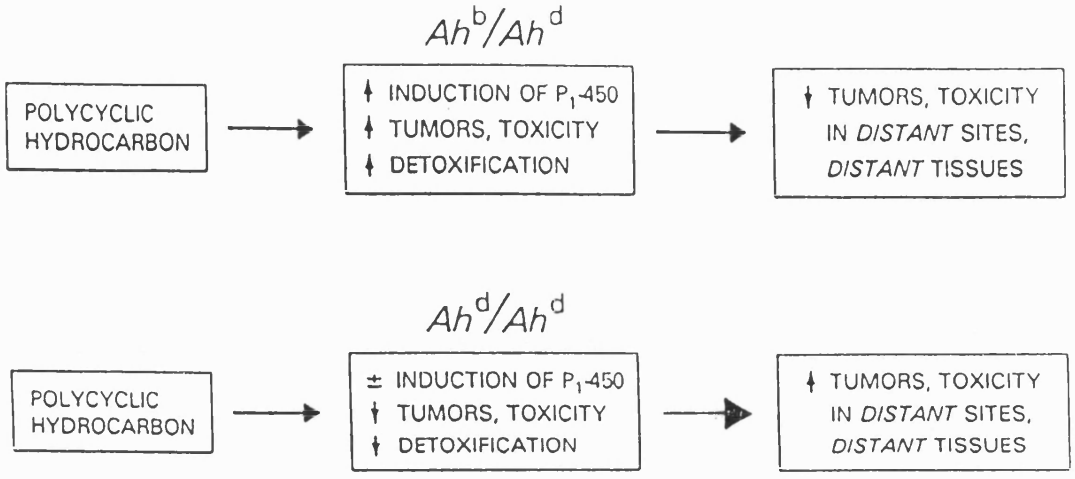


Figure 1.3 - Illustrated scheme indicating the differential susceptibility for tumours in genetically responsive and nonresponsive mice (Nebert, 1981b).

lymphoma, leukaemia and bone marrow toxicity (Nebert et al, 1977; Duran-Reynals et al, 1978; Shum et al, 1979; Nebert & Jensen, 1979; Nebert, 1980b: Nebert, 1980c; Nebert, 1981b; Nebert, 1982).

Relative lack of Ah responsiveness has been attributed to a mutation in the Ah regulatory locus, resulting in the formation of a defective receptor with decreased affinity for the binding of P450IA inducers (Greenlee & Poland, 1979; Nebert et al, 1975; Okey et al, 1979; Negishi & Nebert, 1979).

The human P450IA1 gene has been isolated and sequenced (Jaiswal et al, 1985) and assigned to chromosome 15 (Hildebrand et al, 1985). There is good evidence that polymorphism in P450IA1 activity exists in man (Kellermann et al, 1973b) and that around 10% of the Caucasian population exhibit high P450IA1 inducibility (Kouri et al, 1982). In addition, polymorphism of

the CYPIA1 gene itself has been identified (Kawajiri et al, 1990; Nakachi et al, 1991).

The human P450IA2 gene has also been sequenced (Quattrochi et al, 1986; Jaiswal et al, 1986) and there is some evidence to suggest that certain individuals have higher levels of this enzyme than others (Butler et al, 1989a,b; Bartsch et al, 1990).

1.6 GENETIC POLYMORPHISM AND HUMAN CANCER

The importance of chemical carcinogenesis is demonstrated by the association between human exposure to certain chemicals and the likelihood to develop certain cancers. In this light, the incidence of cancer of the lung (Hammond, 1975) and oral cavity (Muir & Kirk 1960) that has paralleled the increase in cigarette smoking in both sexes is particularly important. In addition, aflatoxin, a mycotoxin common in the African food chain is associated with liver cancer (Peers et al, 1976; Shimada & Guengerich, 1989), asbestos with malignant mesothelioma (Muscat & Wynder, 1991), dye chemicals with bladder cancer (Cartwright et al, 1982) and hair dyes with cancer of the breast (Nasca et al, 1980). Environmental carcinogenesis however, does appear to be modified by the genetic constitution of the host (Bottomley & Conditt, 1968; Anderson, 1975; Mulvihill, 1975; Knudson, 1977; Schimke, 1978; Schull & Weiss, 1982; Blattner et al, 1983). This would explain why upto 20% of lung cancers are not related to smoking habits, in populations where cigarette smoking is widespread; why some nonsmokers may develop lung cancer, while smokers might not and why lung cancer patients frequently have a higher than expected number of relatives with cancer. Heritable risk factors are implicated in cancer aetiology (Yokota et al,

1987; Weston et al, 1989; Caporaso et al, 1990) and first degree relatives of patients with cancer of the lung, colon, stomach and breast have around three times the risk for developing the same cancer than the general population (Anderson, 1975; Burt et al, 1985).

Polymorphic expression of xenobiotic-metabolising enzymes may form the basis for individual susceptibility to chemically induced human cancers (Wolf, 1986; Idle, 1991; Nebert, 1991) in that one phenotype may be more prone than another to develop certain types of cancer.

Subjects with slow NAT activity may be more susceptible to bladder cancer (Cartwright et al, 1982; Evans et al, 1983; Hanssen et al, 1985; Ladero et al, 1985; Vineis et al, 1990) and carcinoma of the larynx (Drozdz et al, 1987). On the other hand, rapid acetylation has been associated with cancer of the breast (Bulovskaya et al, 1978) and with development of colorectal carcinoma (Ilett et al, 1987; Lang et al, 1986), although the latter association has been disputed (Kirlin et al, 1991; Ladero et al, 1991). It may therefore be of value to determine the acetylator phenotype of an individual.

The human debrisoquine polymorphism has also been suggested to be associated with cancer risk. A significantly greater proportion of extensive metabolisers of debrisoquine was found in groups of patients with abdominal cancers associated with aflatoxin in Nigeria (Idle et al, 1981; Ritchie & Idle, 1982), with Balkan endemic nephropathy and renal tumours in Bulgaria (Ritchie et al, 1983) and in patients with carcinoma of the

bronchus (Ayesh et al, 1984; Caporaso & Idle, 1990). Several workers however, have not substantiated the latter finding (Roots et al, 1988; Boobis & Davies, 1990; Speirs et al, 1990; Duche et al, 1991). The poor metaboliser phenotype has been associated with Parkinson's disease (Barbeau et al, 1985).

Increased risk of bronchogenic carcinoma, induced by cigarette smoke, appears to be associated with the high P450IA1 inducibility phenotype in man (Kellermann et al, 1973b) such that the Ah-responsive smoker is 20-30 times more prone to lung cancer than the Ah-nonresponsive smoker (Kouri et al, 1982). Although polymorphism of the CYPIA1 gene has been detected (Jaiswal & Nebert, 1986), most investigators have not linked a particular genotype with increased lung cancer risk (Jaiswal et al, 1985, 1986). However, a recent study has found that more patients with cigarette smoke-induced lung cancer have the rare CYPIA1 genotype corresponding to high P450IA1 activity (Kawajiri et al, 1991).

Cancer of the breast (Pyykko et al, 1991) and tropical chronic pancreatitis (Chaloner et al, 1990) have also been associated with high P450IA1 inducibility in man, while an increased risk of acute leukaemia of childhood may be associated with the low P450IA1 phenotype (Blumer et al, 1979).

As P450IA2 activity is highly correlated with cancer induced by aromatic amines in animals (Kamataki et al, 1983), the human orthologue is of great interest.

Cigarette smoke and especially its particulate matter (tar) contain carcinogens and cocarcinogens (Wynder & Hoffmann, 1979)

and tobacco is generally recognised as being responsible for 80-90% of cancers of the lung and oral cavity (Hammond, 1975). Early diagnosis of bronchogenic, head and neck cancers is unusual (Sanderson & Jett, 1989). As P450IA may activate carcinogens in cigarette smoke, the screening of populations for subjects with high P450IA activity may be useful in determining those who are at high risk for cancer of the lung, head and neck.

The incidence of colorectal cancer is second only to malignant lung tumours and increasing. This disease shows considerable variation in incidence worldwide and like lung cancer, environmental factors are thought to be important in its aetiology (Reddy et al, 1980). Conversely, familial adenomatous polyposis (FAP) is a hereditary disorder, transmitted as an autosomal dominant trait involving chromosome 5 and is determined by a single gene (Harris et al, 1980). It is a clearly defined premalignant lesion of the colon and rectum, with carcinoma developing in 100% of patients if they are followed up for sufficient length of time. Currently, prophylactic colectomy by the age of 20 y offers the only hope of cancer prevention (Barton, 1992).

Although the most potent cause of FAP is the mutant gene, environmental factors may alter the disease process (Utsunomiya et al, 1974). Dietary chemicals may be important in FAP onset, as the pattern of polyps throughout the bowel, parallels mucosal exposure to bile. As dietary arylamines may be activated by P450IA2 and possibly inactivated by NAT, the screening of populations for subjects with low and high P450IA2 and NAT activities, may be useful in determining those who are risk for

certain premalignant disease and cancer.

1.7 IN VIVO ASSESSMENT OF DRUG METABOLISM

The most widely used technique for assessing the activity of the hepatic drug-metabolising enzymes is to study the kinetics of model drug substrates *in vivo* (Park, 1982). An ideal probe drug for assessing hepatic enzyme activities should have the following properties: (1) single compartment pharmacokinetics; (2) elimination entirely dependent on hepatic metabolism; (3) metabolism independent of liver blood flow and protein binding. In addition, drugs administered orally should be rapidly and completely absorbed (Park, 1982).

For measurements involving urinary metabolites as indicators of enzyme activity, (1) the fraction of the dose of drug absorbed should be known; (2) elimination should be first order; (3) the excretion rate should be rapid compared to the rate of metabolism, so that metabolism is the rate limiting step in the appearance of the metabolite in urine; (4) the metabolite should be excreted entirely by the kidneys and (6) the metabolite should be produced exclusively by the enzyme(s) of interest (Park, 1982; Bartsch *et al*, 1982).

Caffeine as a probe drug

Coffee, the principal source of caffeine, is said to be the world's most widely traded commodity after oil and the near universal use of caffeine-containing beverages and foods exceeds the popularity of alcoholic beverages (Dews, 1984). Caffeine is present in coffee, tea, cocoa and chocolate and the cola nut (Tarka, 1982; Barone & Roberts, 1984) and its wide consumption

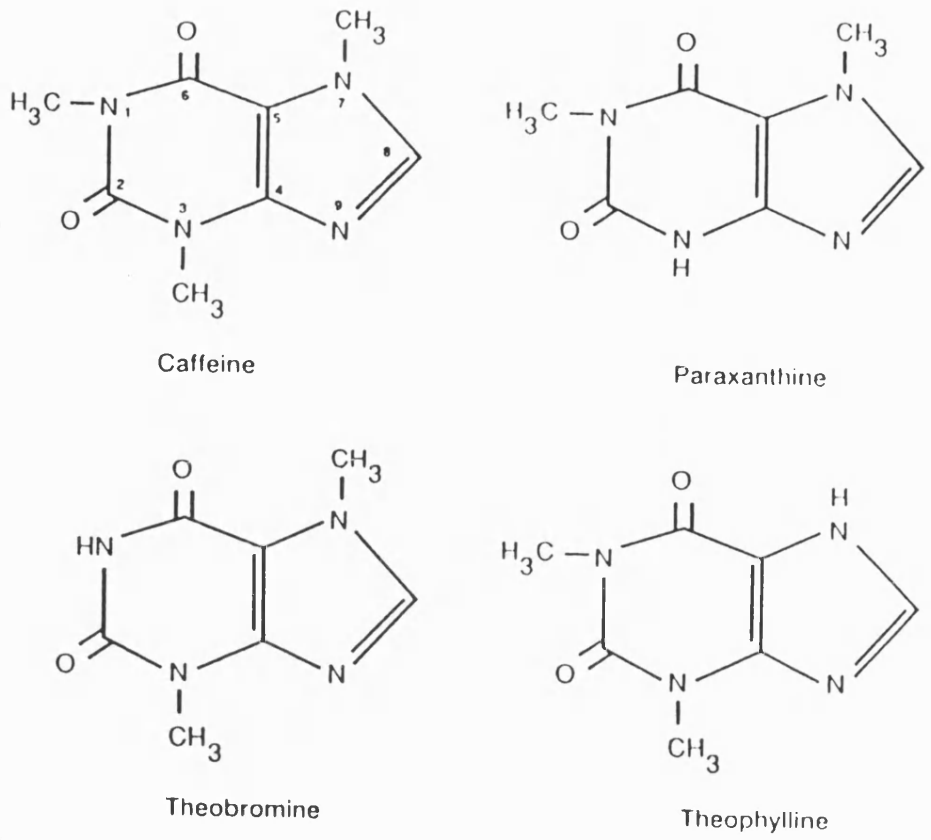


Figure 1.4 - The structure of caffeine and its dimethylxanthine metabolites.

is related to its stimulant properties (Dews, 1984; Gong et al, 1986). The structures of caffeine (1,3,7-trimethylxanthine) and its dimethylxanthine metabolites: paraxanthine (1,7-dimethylxanthine), theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine) are shown in Fig.1.4.

Absorption and distribution.

Following oral ingestion, caffeine is rapidly and essentially completely absorbed form the gastrointestinal tract into the bloodstream (Blanchard & Sawers, 1983). Bonati et al (1982) studied the plasma concentrations of caffeine in healthy subjects and found that following an oral dose of 5 mg/kg, peak levels were achieved in 47 ± 5 min. They reported that 99% of

the administered dose of caffeine was absorbed in approximately 45 min.

Once ingested, caffeine is readily distributed throughout the whole body (Bonati et al, 1982). It is detected in breast milk, placenta and semen and crosses the blood:brain barrier but does not appear to sequester in particular body organs (Yesair et al, 1984).

Elimination

The disappearance of caffeine from plasma in adults and infants is first order and excretion is faster than metabolite formation (Aldridge et al, 1979). In humans, the elimination half-life of caffeine varies between 1.5 and 9.5 h (Bonati et al, 1982) but this may be influenced by a variety of factors. Age (Yesair et al, 1984), use of oral contraception (Patwardhan et al, 1980; Callahan et al, 1983), pregnancy (Aldridge et al, 1979), cigarette smoking (Hart et al, 1976; Parsons & Neims, 1978; Kotake et al, 1982) and hepatic disease (Scott et al, 1989) may alter the elimination of caffeine by affecting its metabolism.

Metabolism

Caffeine is extensively metabolised and only around 1% of the caffeine dose is excreted unchanged in urine (Newton et al, 1981; Tang-Liu et al, 1983). Whereas 85% of the caffeine dose was recovered in urine by Bonati et al (1982) within 24 h, others have recovered only 70% (Arnaud & Welsch, 1981; Tang-Liu et al, 1983). The markedly prolonged caffeine half-life in patients with liver disease (Scott et al, 1989) is evidence that

the biotransformation of caffeine is performed primarily by the liver (Muir et al, 1980).

A number of reports have verified that caffeine undergoes oxidative N-demethylation via the P450IA enzymes and ring hydroxylation by other P450 enzymes, to produce a variety of methylated xanthines and uric acid derivatives (Cornish & Christman, 1957; Wietholtz et al, 1981; Arnaud & Welsch, 1981; Callahan et al, 1982; Tang et al, 1983; Arnaud, 1984). The multiple pathways by which caffeine is known to be metabolised have been established from metabolites identified in urine (Arnaud & Welsch, 1981). Caffeine is metabolised through a series of sequential and competing steps and is virtually completely transformed (Burg, 1975) (Fig.1.5).

Some 70-80% of caffeine is metabolised to paraxanthine (1,7-DMX) in man (Arnaud & Welsch, 1981; Callahan et al, 1982) and 1,7-DMX and its metabolite 1,7-DMU account for 12% of the caffeine dose excreted in urine; theophylline (1,3-DMX) accounts for 3% and theobromine (3,7-DMX) for 2% (Cornish & Christman, 1957; Bonati et al, 1982). 1,7-DMX is formed at about seven times the rate of the other two dimethylxanthines and appreciable quantities of 1,7-DMX (up to 2ug/ml) are detected in the plasma of coffee and tea drinkers (Lelo et al, 1986a). It is surprising therefore that although caffeine consumers are continuously exposed to 1,7-DMX, there is very little information relating to its effects in humans, even though available evidence suggests that 1,7-DMX is more toxic than caffeine in the rat (Bortolotti et al, 1985).

Of the monomethylxanthine and uric acid metabolites, 1-MU and 1-MX are the major compounds assayed in urine and up to 30% of

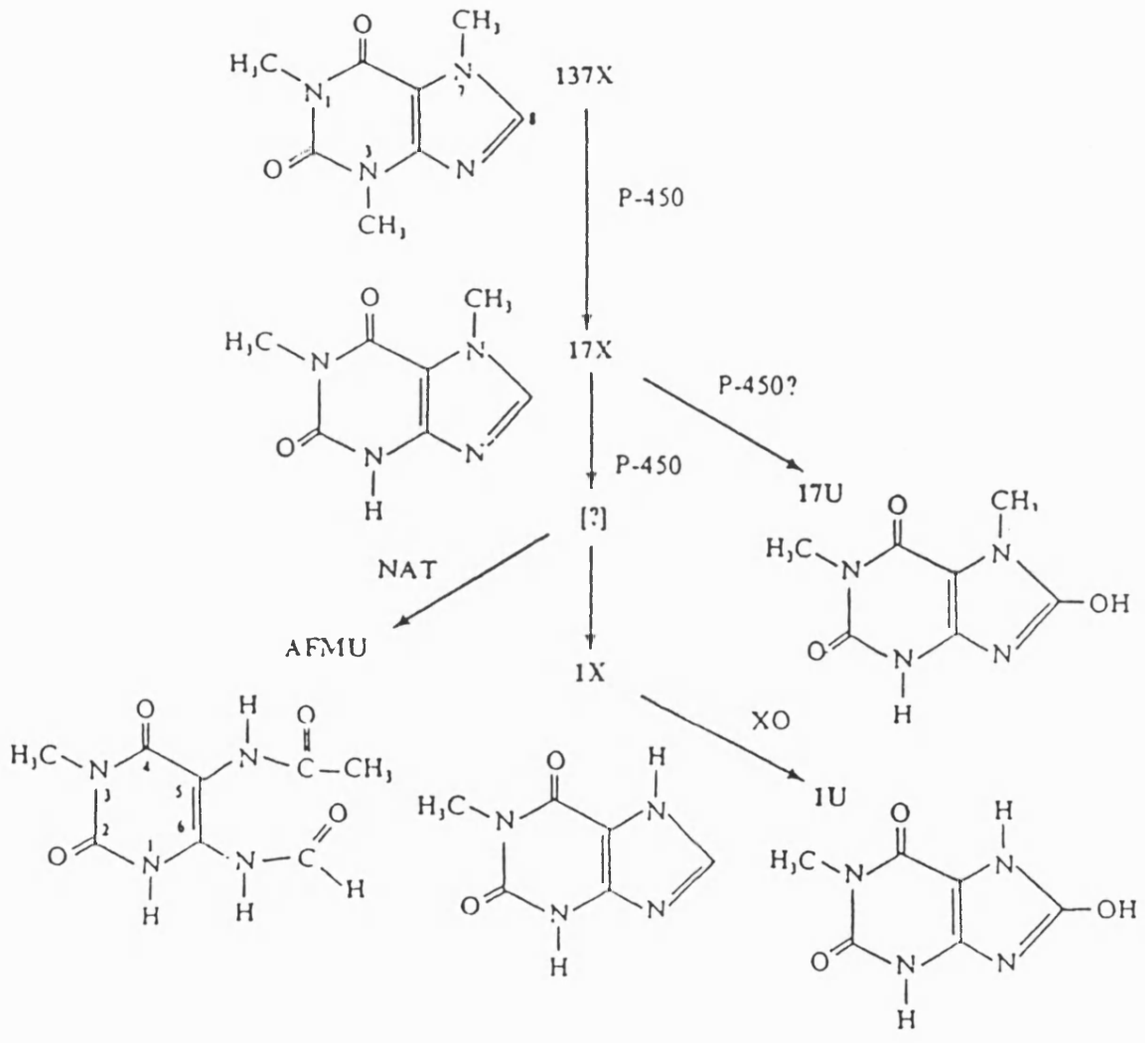


Figure 1.5 - The major metabolic pathway of caffeine in man.

the caffeine dose may be recovered as 1-MU (Bonati et al, 1982). 1-MX and 1-MU were found to account for around 50% of 1,7-DMX clearance in a study by Lelo et al (1989); AFMU for 17% and renal excretion of unchanged 1,7-DMX comprised 9% of its clearance. However, Callahan et al (1982) found that AAMU and AFMU accounted for up to 40% of the caffeine dose.

Caffeine Metabolite Ratios

The profile of urinary metabolites of caffeine in man has been found to vary significantly between individuals (Parsons & Neims, 1978; Aldridge et al, 1979; May et al, 1982; Grant et al, 1983a, Lelo, 1986a,b,c; Campbell et al, 1987; Birkett & Miners, 1991). In order to use caffeine as a probe drug to measure such variation, metabolite ratios (MRs) may be used. The MR is extensively used and is usually expressed as D/M, where D and M are the concentrations of drug and metabolite, respectively in urine. Urinary ratio frequency plots appear to be more discriminatory than those for urinary recovery of a metabolite and differences in metabolic ability are often best determined by MRs as indirect measures of enzyme activity (Jackson et al, 1986). An implicit assumption in the use of the MR is that renal clearance remains constant across populations and that renal clearance is less variable than metabolic clearance (Birkett et al, 1981). As 1-MX, 1-MU and AAMU are very polar compounds, they are likely to be excreted by the kidney without reabsorption. Hence the MR should be relatively insensitive to variation in urine flow or kidney function (Grant et al, 1984).

The advantage of using MRs is that they are essentially independent of the total recovery of the metabolites with time,

but relate the amounts of metabolites measured to each other. Therefore, any variability in a ratio implies variation in an enzyme pathway (Grant et al, 1983b).

There are limitations in the methods for establishing the presumed existence of, or identifying high risk groups of individuals in the population. Firstly, the P450IA enzymes involved in the metabolism of caffeine are inducible by many commonly encountered chemicals, such that intra- and inter-laboratory reproducibility may prove difficult to demonstrate. Secondly, for studies involving patients with lung cancer, it is not known whether hepatic P450IA activity truly reflects P450IA1 activity in the lung - the preferential target organ for tobacco-smoke carcinogens. Lastly, it is conceivable that cigarette smoking and/or malignant states perturb the normally prevailing relationship between enzyme activities in liver and lung (Bartsch et al, 1982).

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Drugs and Reagents

Drugs and reagents used in the present investigation were purchased from Sigma (Poole, UK) and are listed in Table 2.1. Chemicals were of analytical grade purity and were used as rececived without further purification.

5-Acetylamino-6-formylamino-3-methyluracil was kindly gifted by Dr MJ Arnaud (Nestle, La Tour-de-Peilz, Switzerland).

2.1.2 Acids and Salts

Hydrochloric acid; glacial acetic acid and perchloric acid were purchased from Rathburns (Scotland, UK). Sodium hydroxide and ammonium sulphate were of Analar grade and purchased from Sigma (Poole, UK).

2.1.3 Solvents

Acetonitrile and acetic acid used for high performance liquid chromatography were of HPLC grade. Isopropyl alcohol, chloroform and methanol used for these studies were of analytical grade purity. All solvents were obtained from BDH (Bristol, UK). Water used for this study was double-distilled by a 'Fi-Streem' water purification system (Fisons, UK) and subsequently de-ionised (Bibby Products Ltd., UK).

2.1.4 Enzymes

Functionally active human cDNA-expressed cytochrome P450IA1 expressed in yeast cells was a gift from Dr. M.S. Ching

(University of Sheffield). The microsomal pellet was resuspended in 0.1M potassium phosphate buffer (pH 7.4) containing 1mM EDTA and 0.65M sorbitol at a protein concentration of 15 mg/ml. Cytochrome P450IA1 had a specific activity of 43.6 pmol/mg of microsomal protein.

Control yeast cells containing undetectable levels of cytochrome P450 were also prepared in the Department of Pharmacology and Therapetics, University of Sheffield.

2.2 INSTRUMENTATION

2.2.1 High Performance Liquid Chromatography System (HPLC)

HPLC assays were performed using LDC (Laboratory Data Control) components (Staffordshire, UK). The mobile phase was pumped to the column at constant flow by means of a Constametric CM4000 tertiary pump and programmable multiple solvent delivery system. Samples were automatically injected onto the column through a 100ul loop from a rotating Promis I autosampler and the eluted products were detected using a Spectromonitor 3100 variable wavelength U.V. spectrophotometer (UV range 190-350 nm). Chromatograms were produced on a chart recorder and peak heights and areas determined by a CI-10B integrator.

Both the 5 cm long precolumn and the 25 cm long analytical column consisted of stainless steel tubes with a 4.6 mm internal diameter, packed with reversed-phase microparticle silica, 5 um (Spherisorb ODS, Phase Separations Ltd., UK). The equipment was operated at room temperature throughout. Mobile phase was filtered through a 0.45uM membrane filter (Millipore Corporation, UK) before use and subsequently maintained degassed by constant purging with helium (BOC, Bristol).

Table 2.1 - Caffeine, xanthine and uric acid metabolite standards used in the study

Standard	Abbreviation	Storage	Molecular Weight
Caffeine (1,3,7-trimethylxanthine)	1,3,7-TMX	RT	194.2
Paraxanthine 98% Pure (1,7-dimethylxanthine)	1,7-DMX	RT	180.2
Theophylline (1,3-dimethylxanthine)	1,3-DMX	RT	180.2
Theobromine (3,7-dimethylxanthine)	3,7-DMX	RT	180.2
1,7-dimethyluric acid	1,7-DMU	RT	196.2
1,3-dimethyluric acid	1,3-DMU	0-5°C	196.2
3,7-dimethyluric acid	3,7-DMU	RT	196.2
1-methylxanthine	1-MX	RT	166.1
3-methylxanthine	3-MX	0-5°C	166.1
7-methylxanthine	7-MX	RT	166.1
1-methyluric acid	1-MU	RT	182.1
3-methyluric acid 95% pure	3-MU	RT	182.1
7-methyluric acid	7-MX	0-5°C	182.1
8-chlorotheophylline		RT	214.6

2.3 HUMAN STUDIES

All human studies received prior approval from the Bath Health District Research Ethics Committee. A study involving a group of patients with familial adenomatous polyposis (FAP) attending St. Mark's Hospital, London was carried out following approval from the Regional City & Hackney Health Authority.

All control volunteers and patients participating in the caffeine test were of Caucasian origin.

2.3.1 Control Volunteers

Personnel from the Ministry of Defence (MOD), Foxhill, Bath and the Royal United Hospital (RUH), Bath, participated in this study. All were judged healthy following an interview with a Nursing Sister and results of liver and kidney function tests. For these tests, the following measurements were made by the Department of Biochemistry, RUH: plasma sodium, calcium, potassium, bicarbonate, phosphate, urea, total protein, albumin, globulin, bilirubin, alkaline phosphatase and aspartate transaminase. For all studies subject age, body mass index (BMI), current medication and smoking status were recorded (Appendix 1-4). Studies involving control volunteers were classified in three parts.

a) Effect of caffeine consumption on urinary metabolite profiles

Twenty-three members of staff (9 male and 14 female) from the Wolfson Centre, RUH were studied. Their ages ranged between 22 and 56 y (31.07 $\pm$ 1.85; mean age $\pm$ standard error of mean (SEM)). Six volunteers smoked cigarettes; 7 took oral contraceptive steroids (OCS) and none was taking prescribed or

over-the-counter medication. Volunteers participated in several caffeine tests following different protocols:

(i) Seventeen subjects consumed a caffeine containing beverage (CCB) i.e. tea, coffee or cola in the morning, after emptying their bladder. No further CCBs were consumed during the test and all urine passed in the subsequent 8 h was collected for analysis of caffeine metabolites (Appendix 1a).

(ii) The above protocol was repeated two to three months later in ten of the volunteers to assess the reproducibility of the test (Appendix 1b).

(iii) Nineteen subjects consumed a CCB in the morning, after emptying their bladder (time 0). CCBs were also consumed at mid-morning, lunch and tea time until four cups in total had been drunk. 0-8 h urine was collected and assayed (Appendix 1c).

(iv) A spot urine sample was collected 2-6 h after a single CCB in seventeen subjects and caffeine metabolites assayed (Appendix 1d).

b) Effect of timing of urine collection on metabolite profiles

Seven subjects (2 male and 5 female) who participated in protocol (a) were studied in detail for changes in caffeine metabolite profiles over 2 h periods, following different caffeine doses.

(i) The first urine passed in the morning was collected and labelled as specimen t0. A CCB was immediately consumed but all subjects abstained from further beverages containing caffeine during the 8 h test. Urine was

passed every two hours and the samples labelled t2, t4, t6 and t8 h (Appendix 2a).

(ii) The above test was repeated but in addition subjects consumed CCBs at mid-morning, lunch and tea time such that t2, t4, t6 and t8 h urine samples were collected, following multiple caffeine dosing (Appendix 2b).

c) 0-8 hour urinary metabolite profiles: a population study

One hundred and twenty-seven subjects (86 male and 41 female) were studied, consisting mainly of MOD staff. Their ages ranged between 21 and 63 y (37.0 ± 1.07; mean ± SEM). Twenty-five volunteers smoked cigarettes; 14 took OCS or hormone replacement therapy (HRT); 5 were using salbutamol pressurised aerosol inhalers; 2 were prescribed aspirin; 5 were taking atenolol; 2 were taking non-steroidal anti-inflammatory preparations for arthritis and 2 were taking diuretics. No other volunteer smoked or took regular medication.

Each subject emptied their bladder on rising and immediately consumed a CCB. No restrictions were placed on caffeine dosing but all volunteers recorded the number of chocolate bars and CCBs consumed during the study. All urine passed over the next 8 h period was collected and pooled (Appendix 3).

d) 2-6 hour urinary metabolite profiles: a population study

One hundred and fifty subjects (59 male and 91 female) were studied, consisting mainly of hospital staff. Their ages ranged between 17 and 91 y (39.4 ± 1.5; mean ± SEM). Forty-five volunteers smoked cigarettes; 28 took OCS or HRT; 3 were using salbutamol pressurised aerosol inhalers; 1 taking sodium

valproate; 1 taking ranitidine; 2 taking penicillin; 1 taking a diuretic; 1 taking atenolol; 1 taking lorazepam and 2 taking non-steroidal preparations for arthritis. No other volunteer smoked or took regular medication.

Each subject collected a spot urine sample 2-6 h after a CCB. No restrictions were placed on the total number of CCBs consumed on the day of the test nor on the time of day that the urine specimen was collected (Appendix 4).

2.3.2 Patients

Three groups of cancer patients admitted to the RUH, Bath or attending its Outpatient Clinics were studied. A group of patients with familial adenomatous polyposis (FAP) and their first degree relatives attending St. Mark's Hospital, London were also studied. Patients recruited had liver and kidney function values within normal limits and none were known to have metastases or evidence of other neoplasia at the time of diagnoses. Several patients had received chemotherapy, months or years before participating in the caffeine test; several were on courses of medication but patients taking drugs known to interfere with caffeine metabolism or cytochrome P450 enzymes, were excluded from the study.

For all studies a record of subject age, BMI, smoking status, current medication and cancer cell type was made (Appendix 5-8).

a) Caffeine metaolism in patients with carcinoma of the lung, head or neck

Fifty-one patients with carcinoma of the lung, head or neck were studied. Forty-five patients were diagnosed by histology and 6

radiographically. Forty were male, 11 were female and their ages ranged between 47 and 83 y (67.92 ± 1.41; mean ± SEM). Sixteen smoked cigarettes at the time of the test; 4 had never smoked and 31 had smoked previously but had given up the habit for at least 2 months and were therefore classified as nonsmokers for the purpose of this study. Forty-one patients had bronchial carcinoma and 10 had cancer in tissue of the head or neck. Thirty had tumours of squamous cell origin; 10 had tumours of oat cell origin; 1 was of Swan cell type; 2 had adenocarcinoma and 8 tumours could not be cell-typed.

Each patient provided a spot urine sample 2-6 h after a single CCB (Appendix 5).

b) Caffeine metabolism in patients with leukaemia

Fifty-four patients with leukaemia were studied. All patients were diagnosed by blood or bone marrow examination. Twenty-two were male, 32 were female and their ages ranged between 24 and 86 y (61.85 ± 2.13; mean ± SEM). Seven smoked cigarettes at the time of the test; 23 had never smoked and 24 had given up smoking for at least 2 months and were classified as nonsmokers. Twenty-nine patients had chronic leukaemia (23 lymphocytic; 3 granulocytic; 3 myeloid); 8 patients had acute leukaemia (2 lymphocytic; 6 myeloid); 10 patients had lymphoma (4 Hodgkin's; 4 Non-Hodgkin's); 4 had myeloma and 3 had hairy cell leukaemia.

Each patient provided a spot urine sample 2-6 h after a single CCB (Appendix 6).

c) Caffeine metabolism in patients with colorectal cancer

Twelve patients with carcinoma of the colon, rectum or anus were

studied. All patients were diagnosed histologically. Five were male, 7 were female and their ages ranged between 54 and 86 y (69.4 ± 2.8; mean ± SEM). Eight were nonsmokers and 4 had given up smoking for at least 2 months and were classified as nonsmokers. Four had cancer of the colon; 6 had cancer of the rectum; 1 of the anus and 1 of the caecum. One patient had a tumour of squamous cell origin and 11 had adenocarcinoma.

Each patient provided a spot urine sample 2-6 h after a single CCB (Appendix 7).

d) Caffeine metabolism in patients with familial adenomatous polyposis

Thirty-seven patients with endoscopically and histologically confirmed FAP attending St. Mark's Hospital, London were studied. All patients had undergone colectomy, prior to the caffeine test. Twenty-one were male, 16 were female and their age ranged between 19 and 70 y (36.38 ± 2.44; mean ± SEM). Five smoked cigarettes; 27 were nonsmokers and the smoking status of 5 patients was unknown. Each patient took caffeine citrate (300mg) by mouth and all urine passed during the following 8 h was collected and pooled (Apendix 8a).

Sixteen first degree relatives of the patients with FAP were studied. These persons were considered to be at risk for FAP, as they may have inherited the mutant gene from affected parents but none had clinical symptoms of FAP at the time of study. Nine were male, 7 were female and their ages ranged between 15 and 65 y (31.25 ± 4.06; mean ± SEM). Six were cigarette smokers and 10 were nonsmokers. Each patient took caffeine citrate (300mg) by mouth and all urine passed in the following 8h was collected and

pooled (Appendix 8b).

2.4 COLLECTION OF BIOLOGICAL SAMPLES

10 ml blood samples were collected in unheparinised glass tubes for estimation of various enzyme, protein and ion concentrations outlined previously, to determine liver and kidney function. Individual blood samples were allowed to clot at room temperature for 30 min. before being centrifuged at 1500g for 15 min to obtain serum.

Following urine collection, the total volume was recorded and approximately 20ml aliquots were stored.

2.5 STORAGE OF BIOLOGICAL SAMPLES

All samples were stored at -20^{o}C between collection and analysis and were subsequently unfrozen at room temperature. Most samples were analysed within a month of collection.

2.6 ANALYSIS OF CAFFEINE AND METABOLITES IN URINE

Due to the wide range in polarity of the caffeine metabolites, the large variation in the levels of their excretion and the instability of one of the metabolites, four separate protocols were employed to analyse caffeine, its polar metabolites, its nonpolar metabolites and AAMU.

2.6.1 Analysis of caffeine and its nonpolar metabolites in urine

1,7-DMX, 1,7-DMU, and caffeine were assayed in urine samples by high performance liquid chromatography (HPLC). The method was a modification of that reported by Grant *et al* (1983a), using 8-chlorotheophylline as the internal standard.

a) Extraction of caffeine and its nonpolar metabolites

Urine samples were extracted with 10 ml chloroform: isopropanol (95:5 % v/v) as follows.

1 ml of saturated ammonium sulphate solution was added to 1 ml of urine sample in screw top glass tubes and vortex-mixed briefly. Two hundred microlitres of 8-chlorotheophylline (100 ug/ml) were added and the resultant mixture was extracted in 10 ml chloroform: isopropanol (95:5% v/v), shaken for 10 min on a rotating mixer and centrifuged at 2000g for 10 min. The organic layer was transferred into a Z10 glass tube (LIP Ltd.,Yorks.) and evaporated to dryness by centrifugal evaporation under a vacuum at 30^{o}C (Gyrovap, V.A. Howe Comp., London). The residue was reconstituted in 1 ml of acetic acid (0.5%) by vortexing for 15 sec. The solution was pipetted into a vial, injected automatically (100 ul volumes) onto the HPLC column.

Calibration.

Blank urine (i.e. with no detectable concentrations of any caffeine metabolites) was achieved after a single volunteer abstained from caffeine for 120 hrs.

Blank urine was spiked with standard solutions of 1,7-DMU (0-5 ug/ml) and 1,7-DMX (0-40 ug/ml) dissolved in water and processed in the same way as the experimental samples. Standard curves were prepared as described in Section 2.8.

b) HPLC conditions for nonpolar metabolites

Column: separations were carried out using a 5 um Spherisorb ODS, 25 cm * 4.6 mm ID reversed phase column

Mobile Phase: 0.5% acetic acid/ acetonitrile (ACN)

Detection of each injected sample was achieved by means of a gradient elution program. % ACN in mobile phase was 0% for 10 min, 0-36% in 20 min, 36-0% in 0.5 min and 0% for 4.5 min.

Flow Rate: 1 ml/min generated a back pressure of 1900 psi

Detection: 280 nm

Range: 0.02 AUF

Chart Speed: 4 mm/min

All separations were performed at room temperature and the metabolites were identified on the basis of their retention times. Under the above conditions, retention times for 1,7-DMU, 1,7-DMX and internal standard in urine extracts were approximately 24.40, 24.80 and 26.80 min respectively.
A typical chromatogram of 1,7-DMU (3 ug/ml), 1,7-DMX (15 ug/ml) and internal standard (20 ug/ml) extracted in blank urine is depicted in Figure 2.1A. A sample of extracted urine from a healthy volunteer, analysed under these HPLC conditions, produced the chromatogram depicted in Figure 2.1B.

c) HPLC conditions for caffeine

A reversed phase HPLC method for analysing caffeine with a short run time was developed.

Column: separations were carried out using a 5 um Spherisorb ODS, 25 cm * 4.6 mm ID reversed phase column

Mobile Phase: 0.5% acetic acid /acetonitrile (ACN) (75:25% v/v)

Flow Rate: 1 ml/min generated a back pressure of 1800 psi

Detection: 280 nm

Range: 0.02 AUF

Chart Speed: 4 mm/min

The retention times for caffeine and IS under these conditions were approximately 5.30 and 4.50 min, respectively. A typical chromatogram of caffeine (2.5 ug/ml) and internal standard (20 ug/ml) extracted in blank urine is depicted in Figure 2.2A. A sample of extracted urine from a healthy volunteer, analysed by this HPLC method produced the chromatogram depicted in Figure 2.2B.

2.6.2 Analysis of the polar metabolites of caffeine in urine

1-MU and 1-MX were assayed in human urine by HPLC. The method used was a modification of that reported by Grant et al (1983a), using 8-chlorotheophylline as the internal standard.

a) Extraction of the polar metabolites of caffeine

Urine samples were extracted with 10 ml chloroform: isopropanol (50:50% v/v) as follows.

Saturated ammonium sulphate solution (0.25 ml) was added to 0.25 ml of urine sample in screw top glass tubes and vortex-mixed briefly. Two-hundred microlitres of 8-chlorotheophylline (100 ug/ml) was added as internal standard and the resultant mixture was extracted in 10 ml chloroform: isopropanol (50:50% v/v), shaken for 10 min on a rotating mixer and centrifged at 2000g for 10 min. The organic layer was transferred into a glass Z10 tube and evaporated to dryness by centrifugal evaporation under a vacuum at 30^{o}C. The residue was reconstituted in 1 ml of acetic acid (0.5%) by vortexing for 15 sec. The solution was

Figure 2.1 - Chromatograms of A) standard 1,7-DMU (3 ug/ml) and 1,7-DMX (15 ug/ml) in blank urine and B) nonpolar metabolites extracted from urine of a healthy subject, with IS (20 ug/ml).

(1) 1,7-DMU, (2) 1,7-DMX, (3) IS.

Values given are the retention times (mins).

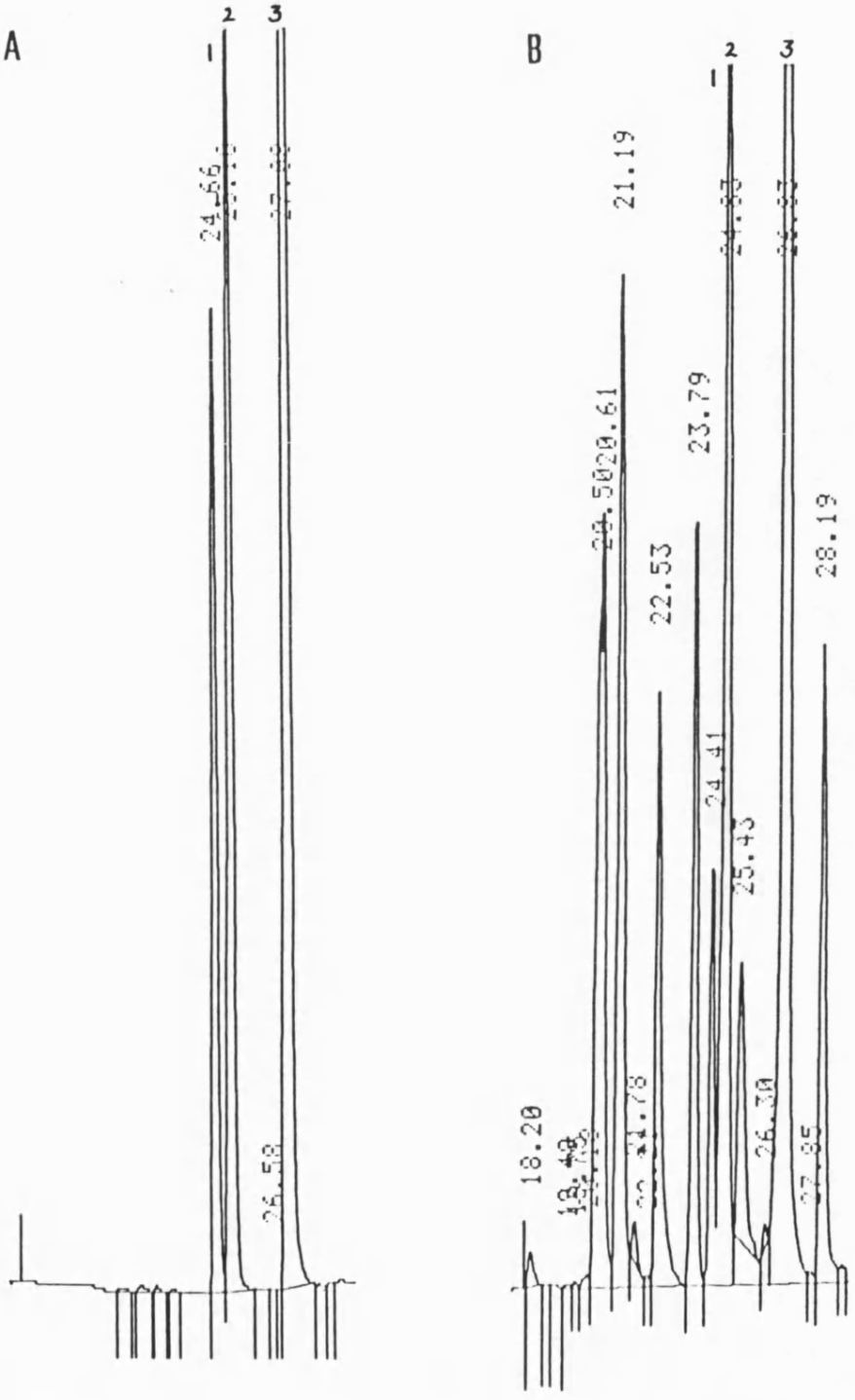


Figure 2.1 - Chromatograms of A) standard 1,7-DMU (3 ug/ml) and 1,7-DMX (15 ug/ml) in blank urine and B) nonpolar metabolites extracted from urine of a healthy subject, with IS (20 ug/ml).

(1) 1,7-DMU, (2) 1,7-DMX, (3) IS.

Values given are the retention times (mins).

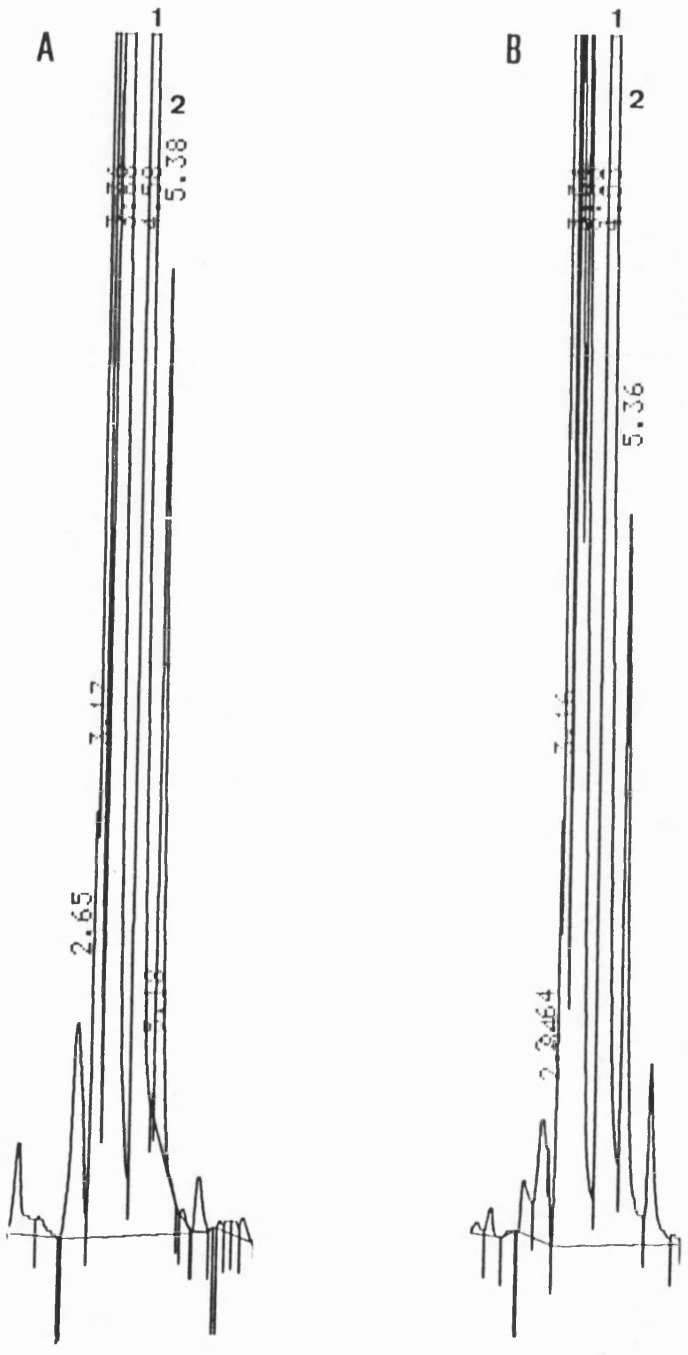


Figure 2.2 - Chromatograms of A) standard caffeine (2.5ug/ml) in blank urine and B) caffeine extracted from urine of a healthy subject, with IS (20 ug/ml).

(1) IS, (2) Caffeine.

Values given are the retention times (mins).

pipetted into a vial, injected automatically (100 ul volumes) onto the column and assayed.

Calibration

Blank urine spiked with standard solutions of 1-MU and 1-MX (0-30 ug/ml) dissolved in 0.001M NaOH solution (pH 9) were processed in the same way as the experimental samples. Standard curves were prepared as described in Section 2.8.

b) HPLC conditions for polar metabolites

Column: separations were carried out using a 5 um Spherisorb ODS, 25 cm * 4.6 mm ID reversed phase column

Mobile Phase: 0.5% acetic acid/ acetonitrile (ACN)
Detection of each injected sample was achieved by means of a gradient elution program. % ACN in mobile phase was 6% for 0-11 min, 6-50% in 6 min, 50-6% in 0.5 min and 6% for 7.5 min.

Flow Rate: 1 ml/min generated a back pressure of 1900 psi

Detection: 280nm

Range: 0.02 AUF

Chart Speed: 4 mm/min

All separations were carried out at room temperature and the metabolites were identified on the basis of their retention times. Under the above conditions, retention times for 1-MU, 1-MX and internal standard in urine extracts were approximately 5.80, 7.70 and 20.40 min respectively.

A typical chromatogram of 1-MU and 1-MX (10 ug/ml) and internal standard (20 ug/ml) extracted in blank urine is depicted in

Figure 2.3A. A sample of extracted urine from a healthy volunteer, analysed under these HPLC conditions, produced the chromatogram depicted in Figure 2.3B.

2.6.3 Analysis of AAMU in urine

a) Assay procedure

Due to the instability of 5-acetylamino-6-formylamino-3-methyl -uracil (AFMU) in urine, this caffeine metabolite was deliberately deformylated into its stable product 5-acetylamino-6-amino-3-methyluracil (AAMU) as follows.

1M NaOH solution (200 ul) was added to 100 ul aliquots of urine and vortex-mixed briefly. The solution was left at room temperature for 10 min and then neutralised with 200 ul 1M HCl. The solution was vortexed again and the resulting 500 ul samples injected directly (100 ul volumes) onto the column. Standard AFMU, kindly donated by Dr MJ Arnaud (Nestle, Switzerland) was dissolved in acetonitrile (100 ug/ml) and aliquots were taken such that a standard curve in the range of 0-40 ug/ml could be produced. Each aliquot was evaporated to dryness and reconstituted in 100 ul blank urine. These standards were then deformylated in the same way as experimental samples.

b) HPLC conditions

A reversed phase HPLC method was developed for analysis of AAMU in urine.

Column: separations were carried out using a 5 um Sperisorb ODS, 25 cm * 4.6 mm ID reverse phase column

Mobile Phase: Acetic acid (pH 2)

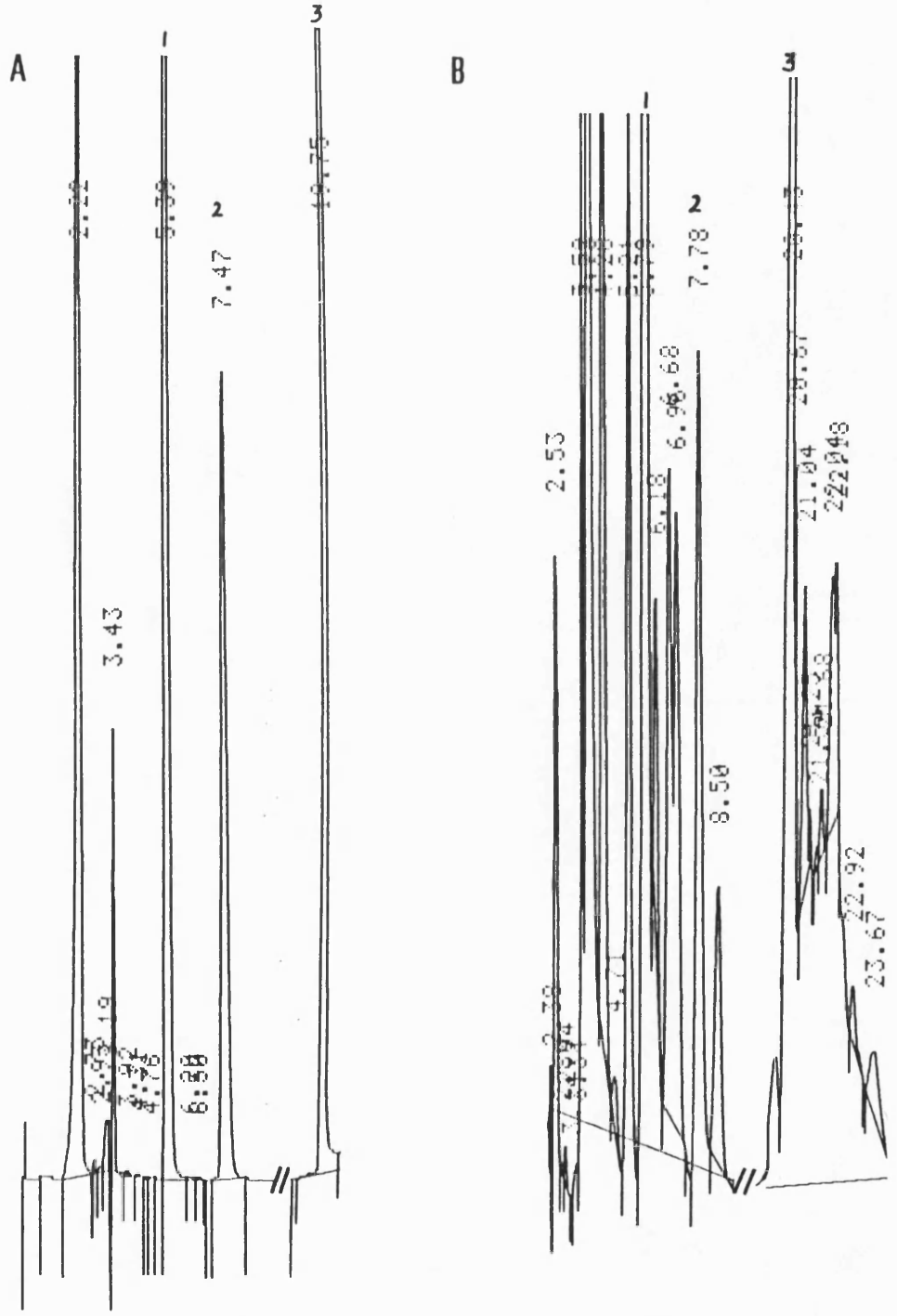


Figure 2.3 - Chromatogram of A) standard 1-MU and 1-MX (10 ug/ml) in blank urine and B) polar metabolites extracted from urine of a healthy subject, with IS (20 ug/ml).

(1) 1-MU, (2) 1-MX, (3) IS.

Values given are the retention times (mins).

Flow Rate: 0.5 ml/min for 0-5 min

1.0 ml/min for 5-8 min

2.0 ml/min for 8-13 min

0.5 ml/min for 13-15 min

Detection: 263.5 nm

Range: 0.05 AUF

Chart Speed: 4 mm/min

AAMU was identified on the basis of its retention time. Under the above conditions AAMU eluted at approximately 7.70 min. Typical chromatograms of standard AAMU (10 ug/ml) in blank urine and AAMU in urine of a healthy volunteer are depicted in Figures 2.4A and 2.4B, respectively.

2.7 IN VITRO METABOLISM OF PARAXANTHINE AND CAFFEINE BY CYTOCHROME P450IA1

2.7.1 Cofactor Solution

For a total incubation volume of 500 ul, the following amounts of cofactors were prepared in order to provide an NADPH regenerating system:

2 umol glucose 6-phosphate

0.2 umol NADP

0.2 units glucose 6-phosphate dehydrogenase

1 umol magnesium chloride.

Cofactors were dissolved in 0.2M potassium phosphate buffer (pH 7.4) to give a total volume of 100 ul.

2.7.2 Standard Incubation Mixtures

For microsomal incubation studies, 50 ug/ml caffeine and 1,7-DMX

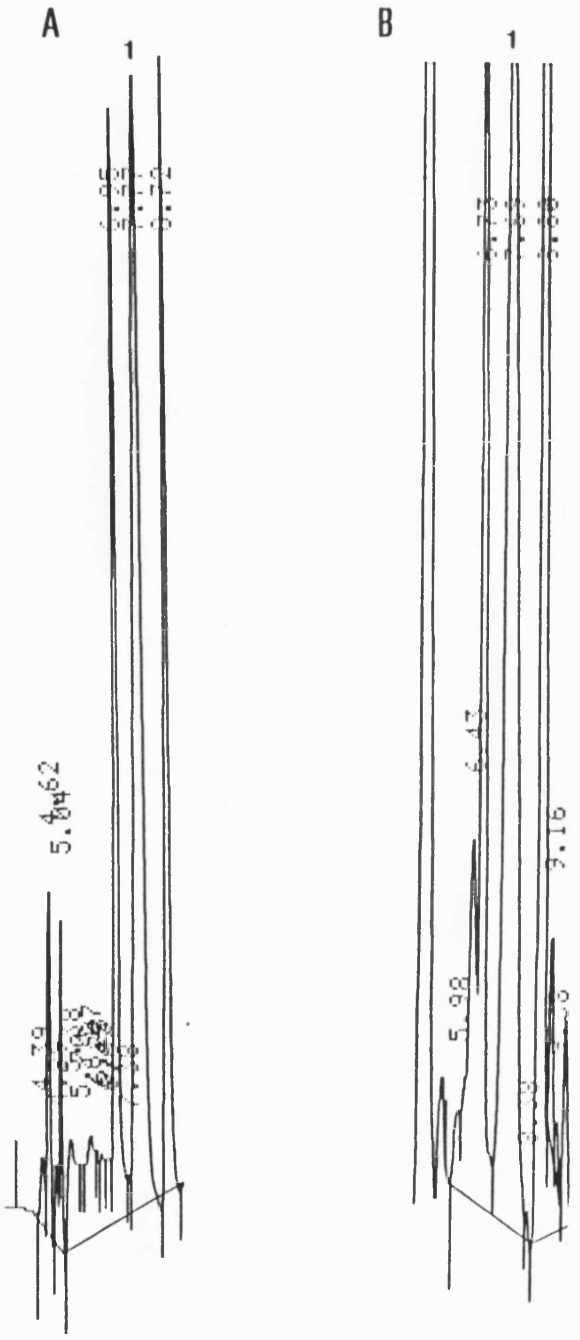


Figure 2.4 - Chromatogram of A) standard AAMU (10 ug/ml) in blank urine and B) AAMU from urine of a healthy subject.

(1) AAMU.

Values given are the retention times (mins).

stock solutions were prepared. Standard mixtures (500 ul) contained 100 ul of the appropriate substrate at a final concentration of 10 ug/ml; 100 ul of cofactor solution and 300 ul of yeast microsomal suspension (15 mgs protein/ml) expressing cytochrome P450IA1, at a concentration of 43.6 pmol/mg protein. Metabolism was initiated by addition of the substrate and the complete mixture was incubated at 37^{o}C for 60 min in a shaking water bath. The reaction was terminated by adding 420 ul of the incubation mixture to 50 ul of perchloric acid (6%), resulting in deproteinization and the protein was sedimented by microfugation at 10,000g for 3 min.

Each incubation was performed in triplicate. Results were compared to control incubations where microsomes expressing P450IA1 were replaced with 300 ul yeast microsomes not expressing P450IA1.

2.7.3 Analysis of caffeine and paraxanthine metabolites

Following protein precipitation and microfugation of each incubation mixture, the supernatant was removed and 50 ul of 8-chlorotheophylline (internal standard; 1 ug/ml) and saturated ammonium sulphate solution (470 ul) was added. The mixture was vortexed briefly.

Incubations with caffeine as substrate were extracted in 6 ml chloroform: isopropanol (95:5% v/v) for analysis of any 1,7-DMX that may have formed. Incubations with 1,7-DMX as substrate were extracted in 6 ml chloroform: isopropanol (50:50% v/v) for analysis of any 1-MX that may have formed. Each mix was shaken, centrifuged and evaporated to dryness as previously described. The residue was reconstituted in 0.5% acetic acid (500 ul) by

vortex-mixing for 15 sec. The sample was pipetted into a vial and injected automatically (100 ul volumes) onto the column. Standard solutions of 1,7-DMX (0-5 ng on column) and 1-MX (0-20 ng on column) with 50 ul internal standard (1 ug/ml; 5 ng on column) were prepared in 300 ul control rat microsomes, made up to 420 ul with water and processed in the same way as the experimental samples.

2.7.4 HPLC conditions

Column: separations were carried out using a 5 um Sperisorb ODS, 25 cm * 4.6 mm ID reversed phase column

Mobile Phase: Acetic acid (0.5%)/ acetonitrile (ACN)
Analysis of each injected sample was achieved by means of a gradient elution program. % ACN in mobile phase was 0% for 0-10 mins, 0-15% in 10 mins, 15% for 5 mins, 15-0% in 0.5 mins and 0% for 9.5 min.

Flow Rate: 1 ml/min for 0-10 min
2 ml/min for 10-25 min
1 ml/min for 25-35 min

Detection: 280 nm

Range: 0.002 AUF

Chart Speed: 4 mm/min

Metabolites were identified on the basis of their retention times. Under the above conditions, retention times for 1-MX, 1,7-DMX, IS and caffeine were approximately 17, 20, 22.50 and 23 mins, respectively. Typical chromatograms of 1-MX (20 ng) and IS

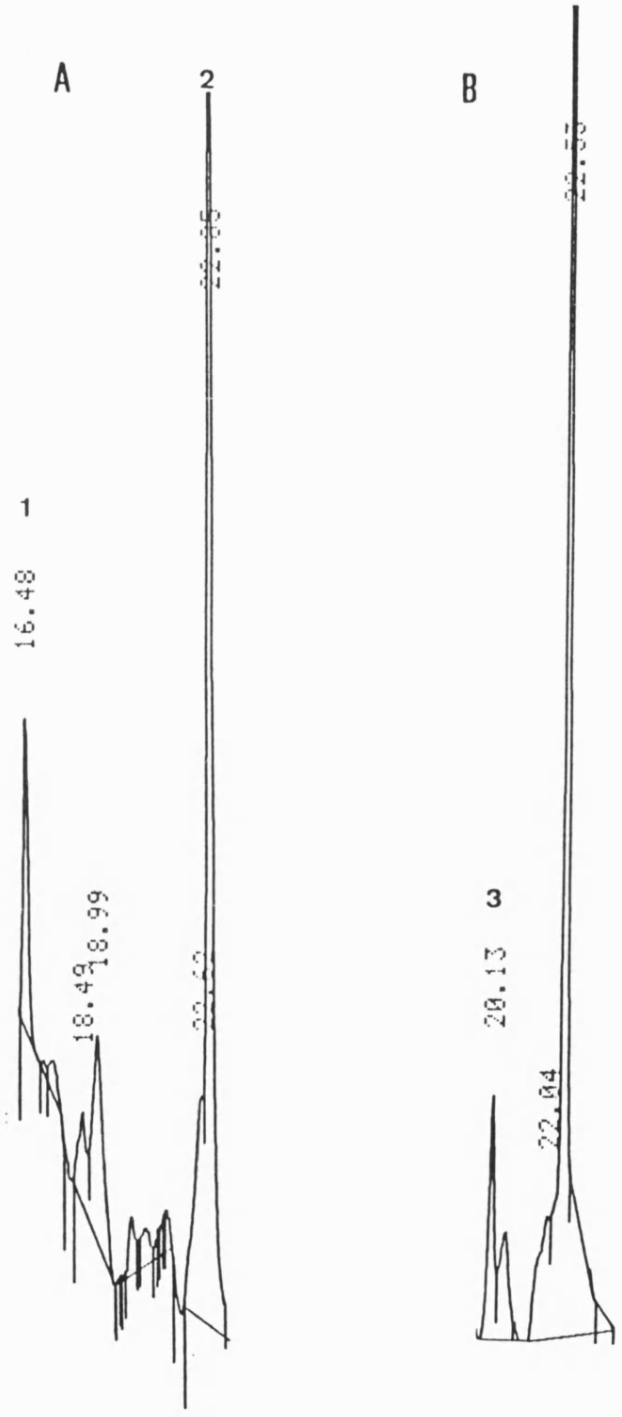


Figure 2.5 - Chromatograms of standard A) 1-MX (20ng) and B) 1,7-DMX (5ng) extracted in control microsomes with IS (5ng).

(1) 1-MX, (2) IS, (3) 1,7-DMX.

Values given are the retention times (mins).

(5 ng) and 1,7-DMX (5ng) and IS (5 ng) in control rat microsomes are depicted in Figs.2.5A and B.

2.8 QUANTITATION OF CAFFEINE METABOLITES IN URINE

a) Preparation of standard curves

Eight to ten blank urine samples were spiked with a range of solute concentrations and a constant concentration of internal standard and assayed by HPLC. Calibration curves for 1-MU and 1-MX, 1,7-DMU and 1,7-DMX and caffeine itself were constructed by determining the peak height ratios (i.e. peak height of metabolite/ peak height of internal standard) of each metabolite concentration in urine. A calibration curve for AAMU was constructed by determining the absolute peak height of varying concentrations in urine.

Any urine samples with a concentration greater than the highest standard(s) were diluted, so as to fall in the linear portion of the calibration curves. Standard curves were prepared for each new batch of urine samples to be extracted/ deformylated. There were no significant changes in gradient with time.

For standard curves of metabolites in microsomal suspensions, lower concentrations were prepared to match those expected to result from incubations. In this way, peak height ratios of absolute ng of each metabolite and internal standard on column were determined.

The data was fitted to the following equation using linear least-squares regression:

$$y = mx + c$$

where y is the peak height ratio or absolute peak height

x is the concentration of solute

m is the gradient and c is the constant (point of crossing the y axis).

b) Linearity of calibration curves

For each standard curve, the correlation coefficient r, the slope m and the intercept c were determined by linear least-squares regression. Typical data for each metabolite calibration curve is shown.

	r	m	c	Range (ug/ml)
Caffeine	0.998	0.0318	-0.0064	0.25 - 20
1,7-DMU	0.998	0.0205	0.0032	0.25 - 5
1,7-DMX	0.999	0.0173	-0.0034	1 - 40
1-MU	0.999	0.0312	0.0097	1 - 30
1-MX	0.999	0.0103	0.0009	1 - 30
AAMU	0.999	3576.6	214.3	0.50 - 40

c) Concentration determination

Peak height ratios for 1-MU, 1-MX, 1,7-DMU, 1,7-DMX and caffeine itself; and absolute peak heights for AAMU were determined in urine. Concentrations were calculated using the equation

$$x_i = \frac{y_i - c}{m}$$

where x_i is the unknown concentration to be determined,

y_i is the observed peak height ratio or absolute peak

height,

c and m are regression coefficients calculated from the calibration curves.

2.9 STATISTICAL ANALYSIS AND TREATMENT OF THE DATA

Statistical analyses of data were carried out using the computer statistical package, Statgraphics (Statistical Graphics Corporation). Results are expressed as mean $\pm$ (SEM). Significant differences between means of different populations were determined by the Student t-test (two-tailed, unpaired) or by non-parametric methods, such as the Mann-Whitney and Wilcoxon tests. Spearman Rank correlations were used to test for goodness of fit of data to a straight line. A probability of $p < 0.05$ was taken to denote a significant difference.

For this study, most of the data are presented in the form of frequency histograms. Skewness of distribution was measured by the Kolmogorov-Smirnov (K-S) test, the Chi-square test and the degree of kurtosis. Statistically significant results for these tests indicated deviation from a normal distribution. In addition to studying cumulative frequency distributions, the Lilly-Fors test was used to determine whether a highly skewed distribution was likely to be bimodal.

A variation of Chi-square was used to test whether the observed frequencies of enzyme activity in a given population were different from the expected frequencies in a control population, based on the following equation:

$$X^2 = \frac{N\,(ad - bc)^2}{(a + b)(a + c)(b + d)(c + d)}$$

	Genetic Trait			
	rr	RR	Rr	Total
Population 1	a		b	a + b
Population 2	c		d	c + d
				N

where a, b, c and d are numbers of individuals expressing a particular genetic trait and N is the grand total of (a + b) and (c + d). A probability of $p < 0.05$ was taken to denote significant difference between observed and expected results.

The absolute quantities of each caffeine metabolite excreted in the urine of different subjects was highly variable. Therefore, all data for comparing individual metabolite profiles were expressed as the percentage of the total metabolites excreted in urine. Total amount (mg) of each metabolite were used to calculate the metabolite ratios which indicated specific enzyme activities.

CHAPTER THREE

THE USE OF CAFFEINE AS AN INDICATOR OF ENZYME ACTIVITY: METHOD DEVELOPMENT

3.1 ANALYTICAL DEVELOPMENT

Introduction

Due to the ubiquitous use of caffeine, its relative safety and its degradation by several enzymes, use of this compound as a probe drug of hepatic enzyme activities warrants investigation. Caffeine is a substance which is metabolised to a large number of different products in man (Arnaud, 1984) and analysis of its metabolites in man is complex. A number of HPLC methods have been developed for the analysis of caffeine and its metabolites in biological samples (Aldridge et al, 1979; Monks et al, 1979; Grygiel & Birkett, 1980; Muir et al, 1980; Callahan et al, 1982; Bonati et al, 1982; Tang-Liu & Riegelman, 1982; Grant et al, 1983b). However, most published methods were inadequate for practical use as they either lacked selectivity or sensitivity; or were unreproducible; or they were elaborate and lengthy. In light of this, the author has developed extraction and HPLC procedures based on modifications of several existing methods.

3.1.1 Extraction procedures

Direct injection of urine samples onto the column was attempted, in order to minimise sample work-up. Unlike other workers (Desiraju & Sugita, 1977), this approach was found to be unsuccessful as endogenous, interfering peaks in urine did not allow adequate resolution of the caffeine metabolites of interest. It was therefore necessary improve test selectivity by

extraction of urine samples with various organic solvents.

Attempts were made to reproduce the extraction procedure described by Aldridge et al (1979) which has been modified by several other groups (Bonati et al, 1982; Grant et al, 1983b; Campbell et al, 1987; Berthou et al, 1989; Kilbane et al, 1990). These workers reported efficient extraction of caffeine metabolites, in a single step using chloroform: isopropanol (85:15% v/v). It was found however, that due to the differences in physical and chemical properties of the mono- and dimethylated xanthines and uric acids, it was not possible to obtain a high extraction efficiency for both groups of metabolites by using a single, extraction step. Therefore two procedures were employed.

The less polar metabolites, 1,7-DMX, 1,7-DMU, 1.3-DMX, 3,7-DMX and caffeine were extracted in chloroform: isopropanol (95:5% v/v). Recovery (%) for the extraction procedure, for each metabolite ranged between 82 and 99.8% and the coefficient of variation between 2.2 and 9.1% (Appendix 9).

Even at low urinary pH, however, when the monomethylated xanthines and uric acids are predominantly in the unionised form, their extraction from aqueous phase into this organic solvent was very inefficient, ranging from 2.8 to 33% by virtue of their high polarity. This seemed to be the case particularly for 1-MU and is in agreement with Muir et al (1980). Methods used by Aldridge et al (1979), Berthou et al (1989) and Grant and coworkers (1983b) extracted 1-MU with % recoveries of 36, 50 and 75%, respectively, which were not deemed satisfactory for this study.

Therefore, 1-MU, 1-MX, 3-MU, 3-MX, 7-MU, 7-MX, 1,3-DMU and

3,7-DMU were extracted in chloroform: isopropanol (50:50% v/v) and by thus increasing the polarity of the organic solvent, the extraction efficiency was increased, ranging from 68.7 to 100% (Appendix 3). In this way 1-MU in particular could be accurately measured from 0-40 ug/ml. The coefficient of variation for these extraction procedures ranged between 4.2 and 7.5% and it was not necessary to resort to ion-pair extraction techniques as previously reported (Muir et al, 1980; Tang-Liu & Riegelman, 1982; Scott et al, 1989).

Additional modifications of the extraction method described by Grant et al (1983b) used in this study were:

a) Substrate volume; for analysis of the less polar metabolites, 1 ml of urine was extracted and for the polar metabolites, 0.25 ml of urine was extracted, in contrast to 0.3 ml.

b) Solvent volume; 10 ml of total extraction solvent were used as opposed to 6 ml to ensure that this was not a limiting factor.

c) Internal standard; 8-Chlorotheophylline was used as internal standard as N-acetyl-para-aminophenol co-eluted with an endogenous peak in urine (Carbo et al, 1989; Bonati et al, 1982; Berthou et al,1989).

d) Use of saturated ammonium sulphate to precipitate proteins in urine. Extraction of caffeine metabolites was enhanced by adding saturated ammonium sulphate solution to the system, in volumes equal to the volume of urine being extracted.

3.1.2 Chromatography

In contrast to several other workers (Aldridge et al, 1979; Berthou et al, 1989; Callahan et al, 1982, 1983; Grant et al,

1983b; Campbell et al, 1987; Carbo et al, 1989) attempts to elute all 14 xanthines and uric acids in one chromatograph were unsuccessful, as peak broadening for compounds eluting late in the run did not allow their accurate quantitation. Grant and coworkers (1983b) could not quantify unchanged caffeine for the same reason. Peak merging also occurred and separation of 1,7-DMX and 1,3-DMX using a single chromatographic procedure was not possible. Furthermore, carry-over peaks from previous injections, representing compounds with long retention times were frequently seen. Therefore gradient elution programs using acetonitrile (Aldridge et al, 1979; Bonati et al, 1982) and acetic acid (0.5%) as mobile phase were developed. Methanol was excluded from mobile phase in the present study, unlike others (Grant et al, 1983b; Grygiel & Birkett, 1980) which allowed better resolution of several peaks.

The gradient system for the nonpolar metabolites (Section 2.6.1) allowed selective measurements of 1,7-DMX, DMMIOD, 1,3-DMX, 3,7-DMX and caffeine whereas previous methods fail to resolve 1,7-DMX and 1,3-DMX (Grygiel et al, 1984; Aldridge et al, 1979; Callahan et al, 1982, 1983) and 1,3-DMU and 3,7-DMX (Tang-Liu & Riegelman, 1982). Although this system allowed quantitative estimation of caffeine itself, this compound was estimated separately and more accurately isocratically, using 25% acetonitrile in the mobile phase so that it eluted at approximately 5.40 min, instead of 30-60 min seen in other methods.

The gradient elution program for the polar metabolites (Section 2.6.2) allowed good resolution of the monomethylated xanthines and uric acids. In the past, this has proved difficult,

particularly for separation of 7-MX and 3-MU (Tang-Liu & Riegelman, 1982), 1-MU and 7-MX (Callahan et al, 1982; Kilbane et al, 1990), 3-MU and 7-MU (Bonati et al, 1982) and 1-MX and 3,7-DMU (Aldridge et al, 1979; Callahan et al, 1982).
Resolution of the rapidly eluting peaks was also achieved when this system was run isocratically with 0.5% acetic acid: acetonitrile (6:94%). In order to maximise quantitative estimation of the peaks however, and to elute endogenous compounds with long retention times, the gradient elution system was used for routine assays. The choice of 280 nm as the detection wavelength optimised the absorbance of both the methyl xanthines (ABS.MAX 270 nm) and uric acids (ABS.MAX 290 nm). Although both groups of compounds have significant absorbance at 254 nm, more interfering and spurious peaks were evident at this wavelength.

It has been established that the portion of AFMU excreted in urine by different subjects is dependent on their arylamine N-acetylation phenotype and simple methods for AFMU analysis were therefore developed (Grant et al, 1983a, 1984). AFMU however, is an unstable caffeine metabolite that is spontaneously deformylated to the stable AAMU (Tang et al, 1983) at ambient temperature at a pH greater than 3.5 (Grant et al, 1984). Most workers have therefore acidified urine samples following their collection in order to stabilise any AFMU present (Hardy et al, 1988; Evans et al, 1989). Due to the fact that AFMU may be retained in the bladder for several hours at a pH and temperature far from ideal for maintaining stability of this metabolite, it would be expected that AFMU recovery may be

low. In fact after 4 h at 37°C, AFMU levels fell by 29% at pH 5; 35% at pH 6; 39% at pH 7 and 45% at pH 8 (Lorenzo & Reidenberg, 1989). Also, Branfman et al (1983) noted that 23% of AFMU degraded in urine frozen for 6 months. AFMU appears to be so labile under physiological conditions that even if urine specimens were adjusted immediately to pH<3 and frozen, there would still be considerable loss of AFMU in more basic urines prior to voiding. This problem was potentiated further by the fact that 1-MX is unstable in aqueous solution if the pH is adjusted to pH<3, in which case it precipitates out of solution (Lorenzo & Reidenberg, 1989). Redissolution of 1-MX in the urine by raising the temperature or pH would accelerate AFMU degradation if both metabolites were measured from the same sample.

It was shown by Tang et al, (1983) that AFMU could be readily and irreversibly changed into AAMU within 10 mins at RT by adding NaOH solution (1M) to the samples and adjusting the pH to 10. As AFMU deformylates quantitatively to stable AAMU, we deliberately deformylated AFMU in urine to its stable product, AAMU. In this way, urine did not need to be acidified; 1-MX recovery remained unaffected; the level of AAMU which represented total acetyluracil formed from paraxanthine may have improved estimation of acetylation capacity.

Initially in the 1960s, the concentration of AAMU in urine was measured by high-performance anion-exchange chromatography but the required time of 40 h per sample was too long for routine usage. It had been reported that AFMU was well extracted in 100% chloroform (Grant et al, 1983b) but we failed to extract AAMU in

this solvent, in agreement with Tang et al (1983) who found that AAMU could not be extracted by chloroform or chloroform/ isopropanol mixtures. In contrast to other workers however (Tang et al, 1986; Kilbane et al, 1990), AAMU was retained by reversed-phase HPLC when mobile phase with pH2 was used and this metabolite could be selectively quantitated in urine as it did not co-elute with any other compounds, even without prior extraction. Exclusion chromatography was thus not used. This method has the advantage of a short run-time (15 min) in comparison with others (Tang et al, 1986).

The detection wavelength was set at 263.5 nm (ABS.MAX for AAMU).

3.1.3 Stability Studies

As each urine sample was stored at -20°C prior to analysis and subsequently unfrozen at room temperature, it was necessary to establish the stability of caffeine metabolites at -20°C over a period of months and at 24°C over a period of hours.

Preliminary results showed that if caffeine metabolites are frozen, they remain stable for at least three months. They also remain stable for over 12 h at room temperature (Appendix 10).

As urine samples were collected for up to 8 h in some caffeine tests, it was also necessary to establish the stability of caffeine and its metabolites at 37°C, as urine may be retained in the bladder at this temperature for several hours.

Results indicated that caffeine metabolites are stable at 37°C for over 10 h (Appendix 10).

3.2 METABOLISM OF CAFFEINE AND PARAXANTHINE BY CYTOCHROME P450IA1

3.2.1 Introduction

It has recently been shown in man that caffeine 3-demethylation to paraxanthine is catalysed exclusively by the P450IA2 enzyme (Butler et al, 1989a; Sesardic et al, 1990). This is the only pathway of caffeine metabolism for which the exact P450 isozyme involved, has been elucidated. One of the major pathways of caffeine metabolism determined during these studies was the 7-demethylation of paraxanthine. A joint study with the Department of Pharmacology & Therapeutics, University of Sheffield (Royal Hallamshire Hospital) was undertaken using human cDNA-expressed cytochrome P450IA1 to determine whether this enzyme was capable of metabolising caffeine and its major metabolite paraxanthine. This approach has been used in the past to determine the substrate specificity of P450IA2 (Aoyama et al, 1989; Gonzalez et al, 1990).

3.2.2 Results

Following incubation of caffeine (10 ug/ml) and 1,7-DMX (10 ug/ml) with yeast microsomes expressing P450IA1, the resultant mixtures were extracted and analysed by HPLC for the presence of 1,7-DMX and 1-MX, as outlined in Section 2.7. These preliminary tests suggested that P450IA1 was capable of metabolising caffeine to 1,7-DMX and that the 1,7-DMX formed during this reaction was itself metabolised further to 1-MX (Fig.3.2.1A). The total conversion of caffeine to these demethylated products was 18.5%, as 5% was metabolised to 1,7-DMX and 13.5% to 1-MX. Control incubations utilising microsomes which did not express

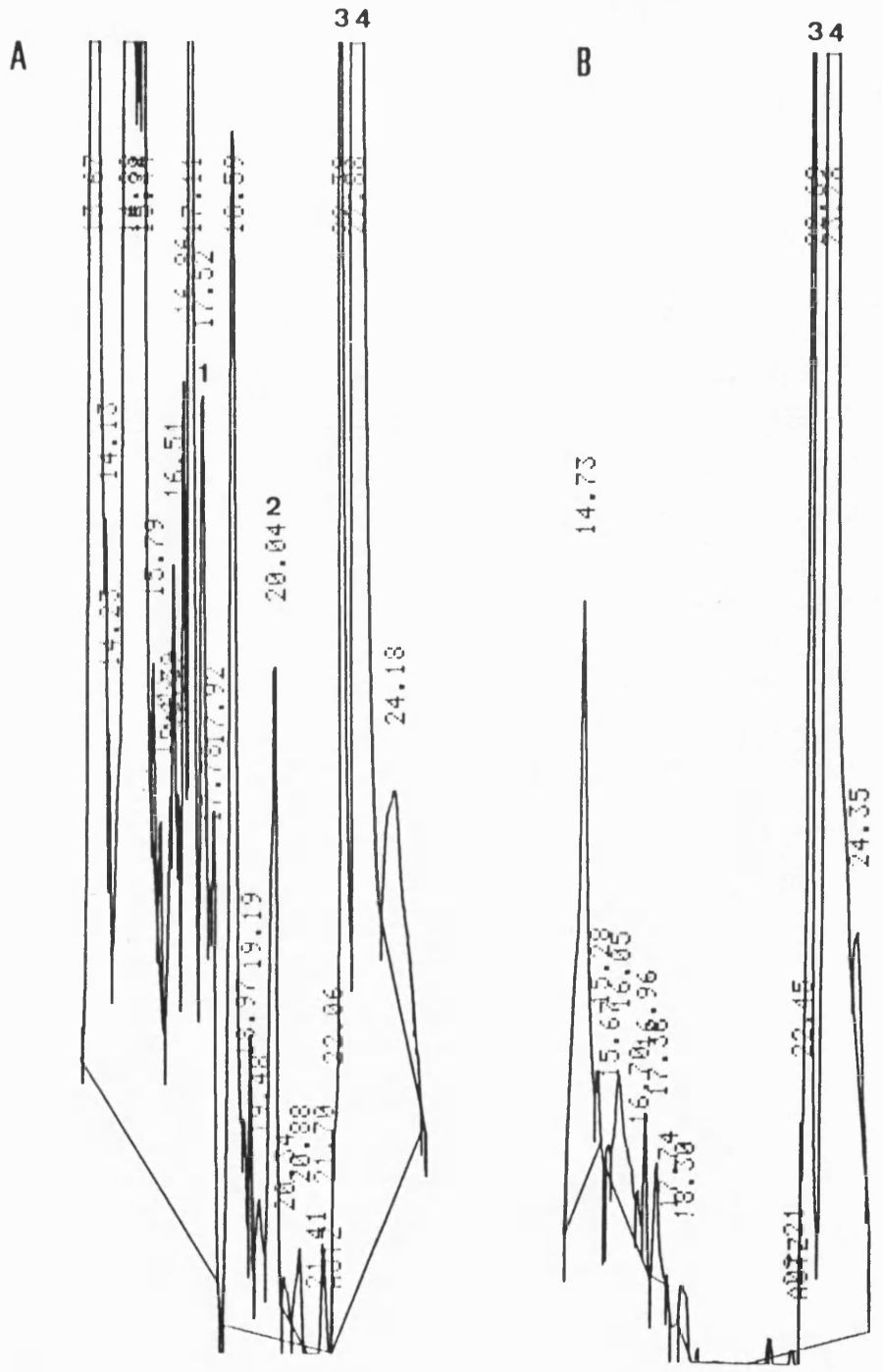


Figure 3.2.1 - Chromatograms of extracted incubates of A) caffeine and microsomes containing P450IA1 and B) caffeine and control microsomes.

(1) 1-MX, (2) 1,7-DMX, (3) IS, (4) Caffeine.

Values used are the retention times (mins).

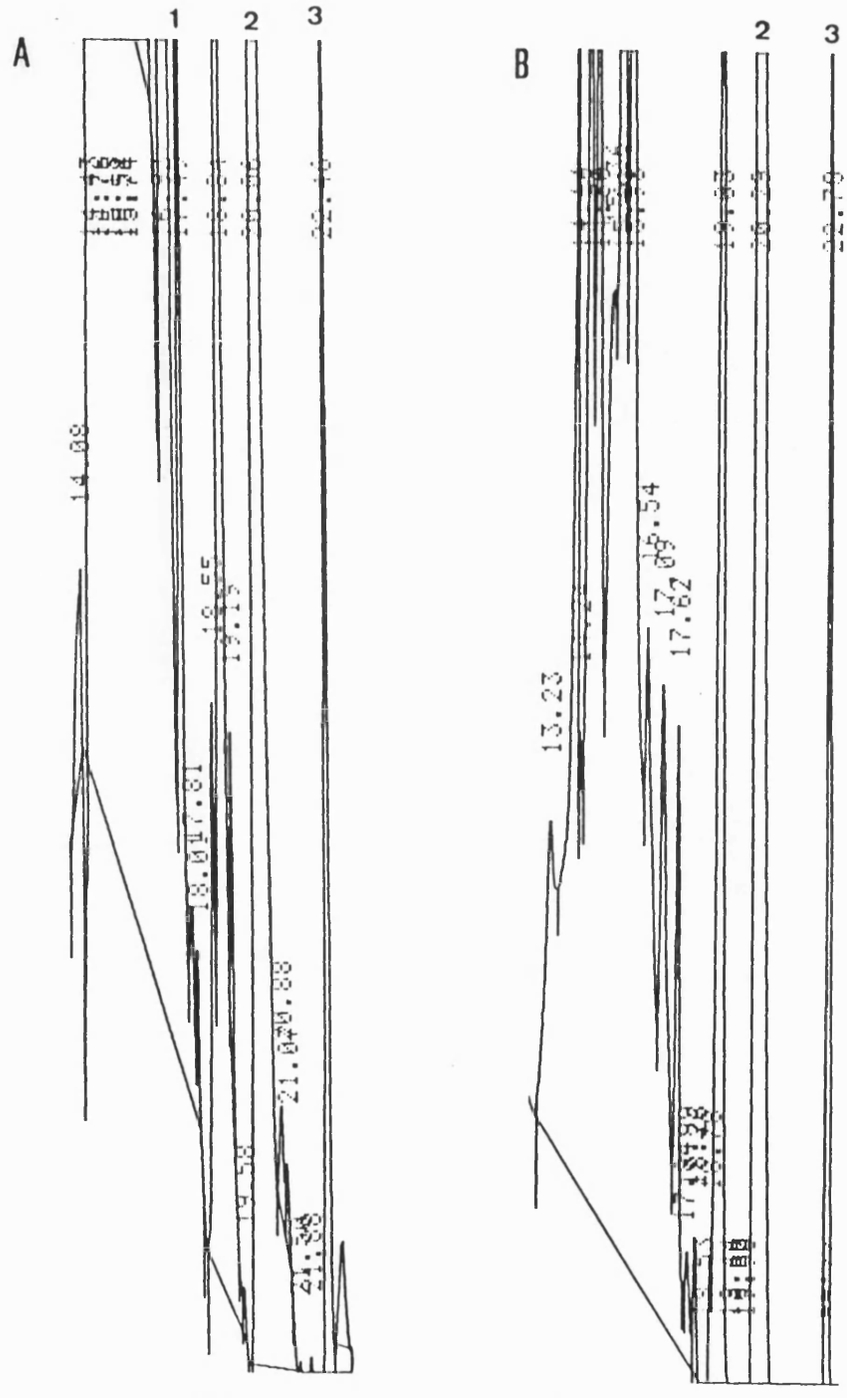


Figure 3.2.2 - Chromatograms of extracted incubates of A) 1,7-DMX and microsomes containing P450IA1 and B) 1,7-DMX and control microsomes.

(1) 1-MX, (2) 1,7-DMX, (3) IS.

Values used are the retention times (mins).

the P450IA1 enzyme, did not metabolise caffeine (Figs.3.2.1B). Microsomes containing P450IA1 also metabolised 1,7-DMX into 1-MX, as shown in Fig.3.2.2A. The total conversion of 1,7-DMX to 1-MX was 49%. Control incubations utilising microsomes which did not express cytochrome P450 enzyme, were unable to metabolise 1,7-DMX (Fig.3.2.2B).

3.2.3 Discussion

Several lines of evidence indicate that N-demethylation pathways involved in the metabolism of caffeine are catalysed by the P450IA family of enzymes, in both animals (Aldridge *et al*, 1977; Welch *et al*, 1977; Bonati *et al*, 1980; Wietholtz *et al*, 1981) and man (Jenne *et al*, 1975; Hunt *et al*, 1976; Parsons & Neims, 1978; Grygiel & Birkett, 1980; Kotake *et al*, 1982; May *et al*, 1982; Grygiel *et al*, 1984; Cusack *et al*, 1985; Miners *et al*, 1985). More recently, studies using human liver microsomes *in vitro* have shown that the N-demethylations of caffeine (Grant *et al*, 1987) and its primary metabolites (Campbell *et al*, 1987) are catalysed by the P450IA family of enzymes. They went as far as suggesting that the same P450IA isozyme mediated all methylxanthine demethylations as pairs of metabolites competitively inhibited the demethylation of each other and biotransformation rates for multiple demethylation pathways were strongly correlated. These workers hypothesised that P450IA1 was responsible for the biotransformation of caffeine (Kalow & Campbell 1988). However, a recent study of caffeine metabolism by Sesardic *et al* (1990) showed that furafylline, a selective inhibitor of P450IA2, inhibited caffeine N3-demethylation. As a previous study by Sesardic *et al* (1988) showed that P450IA1 was

below the level of detection in 45 human liver samples, even in smokers, they concluded that P450IA1 was absent from normal human liver and that caffeine metabolism to 1,7-DMX was catalysed almost exclusively by P450IA2 in man. Wrighton et al (1986) however, found that of 14 human liver samples tested, one specimen did contain P450IA1 and interestingly, this patient had had a clinical history of heavy cigarette smoking. Furthermore, studies by Cresteil & Eisen (1988), Robson et al (1988) and Ikeya et al (1989) show that human liver microsomes contain P450IA1.

In addition, evidence is emerging that metabolism of all the methylxanthines is not the same and that more than one PAH-inducible isozyme is responsible for caffeine metabolism (Bonati et al, 1980; Birkett et al, 1981; Miners et al, 1985; Lelo et al, 1986a; Slusher et al, 1987; Robson et al, 1988; Berthou et al, 1989; Ratanasavanh et al, 1990).

Should P450IA1 be induced in human liver, the preliminary results from this study, suggest that it is capable of metabolising both caffeine and 1,7-DMX to monomethylxanthines. This might be expected to occur because both isozymes of the P450IA family are subject to similar regulatory control and show overlapping substrate specificity. Also, nine demethylation reactions occur during caffeine metabolism and all proceed at different rates, so it seems unlikely that a single enzyme catalyses all these reactions alone.

In the past, liver microsomal preparations were found to metabolise caffeine to its primary metabolites only (Grant et al, 1987; Campbell et al, 1987; Berthou et al, 1988; Berthou et

al, 1989; Sesardic et al, 1990). Elucidation of further pathways in caffeine biotransformation therefore required separate incubations with each dimethylxanthine as the substrate. The present study has shown that incubation of caffeine with human cytochrome P450IA1 enzyme results in the formation of 1,7-DMX and its metabolite 1-MX. Unfortunately, the inhibitor of P450IA2, furafylline was not available, to test whether the purified form of P450IA1 used in these experiments could be inhibited by this compound.

It has also been a common finding that the total percent conversion of caffeine to its metabolites is very low (Lohmann & Miech, 1976; Bonati et al 1980; Campbell et al, 1987; Robson et al, 1988; Berthou et al, 1988; Berthou et al, 1989; Sesardic et al, 1990). For example Grant et al (1987) found that caffeine metabolism never exceeded 1% at a substrate concentration of 1mM for any of the livers that they tested, due to a markedly low turnover rate of the enzyme. The substrate concentrations required in these in vitro studies were therefore considerably higher (0.5-5.0 mM) than those which would be expected in vivo, in order that detectable levels of metabolites could be produced. Low caffeine metabolism in vitro has been explained by incubation conditions being less than optimal. The present results would agree with this in part, as a particularly high degree of caffeine metabolism by the P450IA1 enzyme was not found, but compared to liver microsomal preparations the degree of metabolism was considerably higher. This might suggest that steps involved in actual microsomal preparation are less than optimal. It is known that cDNA expression of P450 enzymes offers numerous advantages over standard approaches in which the P450s

are purified and reconstituted (Gonzalez et al, 1990).

Due to the abnormally low rate of caffeine biotransformation by microsomal preparations, the studies using large substrate concentrations described, have had to assume that their results could be extrapolated to the lower concentrations that would be found in man and interpretations must therefore be approached with caution. This would appear to apply particularly for studies using specific inhibitors of enzyme activities during incubations, as the metabolism of caffeine by control microsomes alone is low enough to make detection of its metabolites a problem. Nonetheless, qualitatively, the metabolites formed in vitro in most studies corresponded well to those detected in the body fluids of man after caffeine intake. Quantitatively speaking, one series of incubation studies with human liver microsomes produced less caffeine metabolites than the others (Sesardic et al, 1990) even though they used 1 mM caffeine as substrate and carried out the incubations for at least 15 mins, like other workers.

In addition to the greater degree of metabolism obtained with yeast microsomes expressing human P450IA1, the present HPLC methods for detecting the metabolites of interest were more sensitive than those used by other workers. The limits of detection for 1,7-DMX and 1-MX were 5ng and 25ng on column, respectively, in comparison to the method used by Ratanasavanh et al (1990) which had a detection limit of 25ng of each metabolite on column. This enabled smaller substrate concentrations (10 ug/ml) to be incubated, which are closer to those amounts found in human tissues following caffeine consumption (Lelo et al, 1986a).

Results from these preliminary studies indicate that should P450IA1 be induced in human liver, it may be able to metabolise both caffeine and 1,7-DMX into their demethylated products.

3.3 CAFFEINE METABOLITE RATIOS AS INDICATORS OF ENZYME ACTIVITIES

3.3.1 P450IA2 Activity

It has recently been demonstrated that the major route of caffeine metabolism via the 3-demethylation pathway to form paraxanthine is catalysed by P450IA2 (Sesardic *et al*, 1990; Butler *et al*, 1989a). Of the nine possible demethylation pathways involved in caffeine metabolism, this is the only route for which the exact P450IA isozyme has been elucidated.

The amount of caffeine excreted compared to its metabolites is low - generally about 1% of the dose (Cornish & Christman, 1957; Burg, 1975; Aldridge *et al*, 1979; Callahan *et al*, 1982; Tang-Liu *et al*, 1983) due to its extensive metabolism in the liver (Bonati *et al*, 1982). The discriminating power of metabolite ratios is therefore high, as slight differences in caffeine metabolism between subjects might be expected to produce large differences in MR values in the population.

Products of the 3-demethylation of caffeine were therefore measured and compared to concentrations of caffeine itself in urine samples. In this way, the following metabolite ratio was determined as an *in vivo* indicator of P450IA2 activity (Fig.1.5):

$$(1,7\text{-DMX} + 1\text{-MX} + 1\text{-MU} + \text{AAMU}) / \text{Caffeine}$$

A similar MR has been used by other workers to determine P450IA2

activity (Bartsch et al, 1990). Any 1,7-DMX which has been subsequently metabolised is accounted for by measuring its major products 1-MX, 1-MU and AAMU. Demethylation of caffeine and the dimethylxanthines is not thought to go beyond the formation of monomethylxanthines because xanthine and uric acid themselves do not accumulate in urine (Cornish & Christman, 1957).

3.3.2 P450IA Activity

In man, paraxanthine formation has been found to account for 70% (Bonati et al, 1982) and 80% (Lelo et al, 1986b) of caffeine metabolic clearance via 3-demethylation. N7-Demethylation of 1,7-DMX to 1-MX is catalysed by the P450IA family of enzymes and has been reported to be the major biotransformation pathway of 1,7-DMX (Lelo et al, 1989). Whether the isozyme involved is P450IA1 or IA2 remains to be determined but this demethylation pathway exhibits the largest response to enzyme inducers and inhibitors and should provide the best measure of induction by cigarette smoke (Grant et al, 1984). 1-MX, 1-MU and AFMU have been found to account for 67% of 1,7-DMX clearance and renal excretion of unchanged 1,7-DMX comprises 9% of its clearance (Lelo et al, 1989). Approximately 50% of the 1,7-DMX dose was excreted as 1-MU and 1-MX and approximately 17% excreted as AFMU in their study, whereas Callahan et al (1982) found that AFMU and AAMU accounted for up to 40% of the caffeine dose. 1,7-Diaminouracil formation has been reported (Arnaud & Welsch, 1981) to comprise around 4% of urinary 1,7-DMX metabolites; this compound was not measured in the present study as an authentic standard was not available. Major products of the

7-demethylation of 1,7-DMX were measured and compared to concentrations of 1,7-DMX itself in urine samples (Fig.1.5). In this way the following metabolite ratio was determined as an *in vivo* index of P450IA activity, based on that from the study by Grant *et al* (1984):

$$(1\text{-MX} + 1\text{-MU} + \text{AAMU}) / 1,7\text{-DMX}$$

7-MU and 7-MX metabolites are not included in the MRs as their low levels of urinary excretion do not allow their accurate quantitation (Grant *et al*, 1984). The validity of this urinary ratio rests on two assumptions. First, although 1-MX formation from caffeine is the sum of 1-MX formed via 1,3-DMX 3-demethylation and 1,7-DMX 7-demethylation, it is assumed that 1-MX formed via 1,3-DMX is negligible, given that <5% of caffeine is metabolised to 1,3-DMX (Cornish & Christman, 1957; Lelo *et al*, 1986b) and that only about 15% of 1,3-DMX is metabolised to 1-MX. Second, it is assumed that the urinary ratio is unaffected by variations in urine flow. The polar nature of 1-MX, 1-MU and AAMU mean that these compounds are likely to be rapidly excreted without reabsorption by the kidney (Tang-Liu *et al*, 1983). Formation of all four metabolites included in the above ratio showed no dependence on total urine volume and consequently a ratio based on these metabolites should show no dependence on urine volume (Section 4.1).

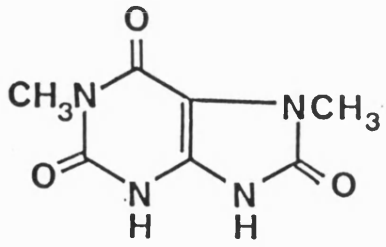
Any 1-MX which has in turn been metabolised, is accounted for by measuring its product 1-MU. Also, the unknown, unstable ring-opened metabolite of 1,7-DMX can be metabolised to form 1-MX or AAMU depending on the acetylation status of an individual (Grant *et al*, 1984; Lelo *et al*, 1989). For this

reason, AAMU is also included in the MR to give as good an estimate as possible for P450IA activity.

As appreciably larger quantities of 1,7-DMX than caffeine are recovered in urine, one may expect an improvement in the reproducibility of measurements using this ratio at the cost of a slight reduction in discriminating power. Furthermore, because the substrate for this ratio is 1,7-DMX and not caffeine itself, results should not be affected by differences in absorption of the test drug.

It should be acknowledged at this stage that 1,7-DMU is a metabolite of 1,7-DMX - albeit a minor one, accounting for around 8% of 1,7-DMX clearance (Lelo et al, 1989). It was discovered late in these studies that the 1,7-DMU standard purchased from Sigma was not authentic. A compound with a very similar structure, called 1,3-dihydro-1-methyl-1-5-(methylamino) -8-imidazo [4,5-d] [1,3] oxazine-2,7-dione (DMMIOD) had been measured (Fig.3.3.1).

A



B

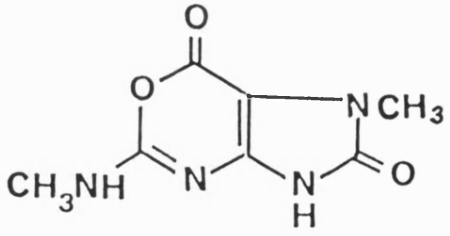


Figure 3.3.1 - Chemical structures of (A) 1,7-dimethyluric acid (1,7-DMU) and (B) 1,3-dihydro-1-methyl-1-5-(methylamino)-8-imidazo [4,5-d] [1,3] oxazine-2,7-dione (DMMIOD).

Other workers have suggested measuring the ratio:

AFMU + 1-MU + 1-MX/ 1,7-DMU as a measure of P450IA1, following the observation that it corelated well with caffeine clearance (Campbell et al, 1987). However, it is unlikely that this ratio reflects P450IA1 activity alone, as P450IA2 is known to be involved in caffeine 3-demethylation in human livers (Sesardic et al, 1990). Furthermore, 8-hydroxylation pathways are not inducible (Brown et al, 1988) and are thought to be catalysed by P450 enzymes other than the IA family, that is, by enzymes that probably do not activate compounds into ultimate carcinogens. Whereas the degree of caffeine and 1,7-DMX demethylation are thought to parallel activation of certain environmental pollutants, it has never been shown that the degree 8-hydroxylation of caffeine is related to some major inactivation pathway of importance in protecting against environmental carcinogens. In the absence of such evidence 1,7-DMX demethylation alone was assayed as a measure of absolute P450IA activity. The lack of 1,7-DMU in the calculation is not therefore believed to undermine the results.

3.3.3 Xanthine Oxidase

Xanthine oxidase is a cytosolic enzyme, responsible for metabolising 1-MX to 1-MU whether its source is paraxanthine (Grant et al, 1983a, 1984, 1986; Lelo et al, 1989) or theophylline (Lohmann & Miech, 1976). Allopurinol pretreatment markedly decreases 1-MU excretion and enhances 1-MX excretion without changing the combined recovery of these metabolites (Grant et al, 1984; Lelo et al, 1989). 7-MX can also be metabolised by this enzyme to form 7-MU (Miners et al, 1982). In

contrast, 3-MX and the dimethylxanthines are not substrates for this enzyme. It is presumed therefore that the following MR can be used as an *in vivo* index of xanthine oxidase activity (Fig.1.5):

1-MU /1-MX

Brown *et al* (1988) also used this ratio as an index of xanthine oxidase activity.

3.3.4 N-Acetyltransferase Activity

Following the isolation (Fink *et al*, 1964) and identification by HPLC (Callahan *et al*, 1982) of a urinary caffeine metabolite, AAMU, it was subsequently shown that AAMU and the major uracilic metabolite, AFMU (Tang *et al*, 1983; Branfman *et al*, 1983) were excreted in urine in varying concentrations, depending on acetylator phenotype (Callahan *et al*, 1983). It has been proposed that an unstable ring-opened intermediate which arises from 1,7-DMX can either be acetylated by the cytosolic NAT enzyme to form AFMU or reclose to form 1-MX (Grant *et al*, 1984; Lelo *et al*, 1989). AFMU and 1-MX are therefore apparently derived from a common intermediate, the formation of which is mediated by P450IA. Urinary concentration of AFMU was found to directly relate to N-acetyltransferase (NAT) activity (Grant *et al*, 1983a) and led to the determination of acetylation phenotype by use of the urinary ratio of AFMU/ 1-MX (Grant *et al*, 1984; Gascon *et al*, 1987; Hardy *et al*, 1988; Evans *et al*, 1989). As discussed in Section 3.1 however, this method is subject to at least two sources of error: the instability of AFMU and assumptions about the mode

and constancy of formation of 1-MX (Tang et al, 1986).

AFMU was deliberately deformylated in urine samples to stable AAMU, overcoming stability problems (Section 2.6.3). Furthermore, 1-MU was measured so that any fluctuations in xanthine oxidase activity were accounted for. The following metabolite ratio was therefore determined (Fig.1.5), as an in vivo indicator of NAT activity, in agreement with Tang et al (1987) and Lelo et al (1989):

$$AAMU/ (AAMU + 1\text{-}MX + 1\text{-}MU)$$

Use of the metabolite/(metabolite + drug) ratio was suggested by Inaba et al (1980) in order to normalise for variability in recovery of unchanged drug. This ratio has several advantages: it is not influenced by the amount of methylxanthine intake in that it does not change following single dose or multidoses of caffeine (Tang et al, 1987); it is independent of xanthine oxidase activity because the sum of 1-MU and 1-MX is included in the denominator; use of this ratio would make sense even if 1-MX is formed directly from paraxanthine without going through a ring-opened intermediate as currently proposed; pH of urine is unimportant because AAMU as the deformylated product of AFMU represents total acetyluracil, formed from paraxanthine.

The validity of this ratio as an index of acetylation phenotype has been demonstrated by the complete accordance with the plasma index for sulphamethazine (SMZ) acetylation (Tang et al, 1987) and dapsone acetylation (Kilbane et al, 1990) in contrast to the AFMU/1-MX ratio previously used (Grant et al, 1983a).

The main disadvantage of the ratio AAMU /(AAMU + 1-MX + 1-MU) compared with AFMU /1-MX is the reduced numeric difference

between values characterising rapid and slow acetylators. The new ratio therefore seems to bring about improvement in measurement at the cost of a slight reduction in discriminating power.

CHAPTER FOUR

THE USE OF CAFFEINE AS AN INDICATOR OF ENZYME ACTIVITY: ROBUSTNESS OF THE TEST

Introduction

Initial caffeine tests were performed involving pooled urine collections as reported by other workers (Cornish & Christman, 1957; Tang-Liu & Riegelman, 1982; Grant et al, 1983a; Campbell et al, 1987; Carbo et al, 1989; Jonkman et al, 1991). Unlike other test drugs, it proved difficult to restrict participants from consuming caffeine over an eight hour period. It was desirable in this study therefore, to measure caffeine and its metabolites in healthy volunteers without restricting caffeine consumption. To this end, 0-8 h urinary metabolite profiles resulting from a single CCB or several CCBs were compared.

Collection of urine samples at outpatient clinics, would necessitate spot urine collection, if there was to be opportunistic enrollment of suitable patients. Spot urinary metabolite profiles were therefore compared in 2 hourly samples and results correlated with 0-8 hour results to see whether a reduction in collection period could be achieved without sacrificing the result.

Procedure

23 subjects, comprising nine males and fourteen females were studied for caffeine metabolism (Section 2.3.1). Caffeine, 1,7-DMX, 1-MX, 1-MU and AAMU were assayed in urine by HPLC as previously described (Sections 2.6). The caffeine metabolite

DMMIOD, originally thought to be 1,7-DMU was also assayed. Details of volunteers and measurements of individual metabolites in urine are given in Appendices 1-2.

4.1 EFFECT OF CAFFEINE CONSUMPTION ON URINARY METABOLITE PROFILES

4.1.1 Metabolite profile in 8 h urine, following consumption of a single CCB (Test 1).

Seventeen healthy subjects (volunteers #1-17) with a mean age of 31.12 ± 2.12 y consumed a single CCB on rising, after an overnight fast. The major metabolites assayed in 0-8 h urine samples were AAMU, 1-MU and 1-MX. 1,7-DMX, DMMIOD and caffeine were also measured (Table 4.1.1) (Appendix 1a).

Table 4.1.1 - Total mgs of caffeine metabolites in 0-8 h urine samples following consumption of a single CCB (n=17)

Metabolite	Total mg (mean ± SEM)	Range
AAMU	38.37 ± 9.72	17.3 - 190.0
1-MU	19.58 ± 2.94	8.6 - 59.2
1-MX	11.79 ± 1.58	5.9 - 26.4
1,7-DMX	3.64 ± 0.39	1.5 - 7.0
*DMMIOD	0.89 ± 0.19	0.2 - 3.6
Caffeine	0.62 ± 0.21	0.1 - 3.0

* Originally thought to be the caffeine metabolite 1,7-DMU.

In order to make direct correlations between the metabolites measured in different caffeine tests, recovery (%) of each metabolite was also determined. The metabolites listed in Table 4.1.1 were presumed to account for 100% of the total caffeine metabolites in urine as other measurable metabolites, such as 7-MX, were present in smaller quantities. Recoveries (%) for each caffeine test are compared in Fig.4.1.1. When the metabolites of caffeine were compared, AAMU correlated negatively with 1-MX ($r=-0.777$, $p<0.005$) and with 1-MU ($r=-0.652$, $p<0.01$). There was also a tendency for DMMIOD and 1-MX to correlate negatively ($r=-0.434$, $p=0.08$). None of the metabolites was shown to correlate with total 0-8 h urine volume.

4.1.2 Intra-subject Reproducibility (Test 2).

The test involving consumption of a single CCB and collection of 0-8 h urine was repeated two to three months later in ten subjects (#2,3,6,7,8,9,12,13,16,17). Total mgs of each metabolite measured are shown in Table 4.1.2 (Appendix 1b). The major metabolites recovered in urine were similar to that of the first test as shown in Fig.4.1.1.

The % recoveries of AAMU and 1-MX ($r=-0.9273$, $p<0.01$) and AAMU and 1-MU ($r=-0.6364$, $p=0.0563$) were found to correlate in a similar way as they did for the first test. In this repeat test, % recoveries of 1,7-DMX and DMMIOD ($r=0.7212$, $p<0.05$) and caffeine and 1,7-DMX ($r=0.8667$, $p<0.01$) were also found to correlate. None of the metabolites was shown to correlate with total urine volume.

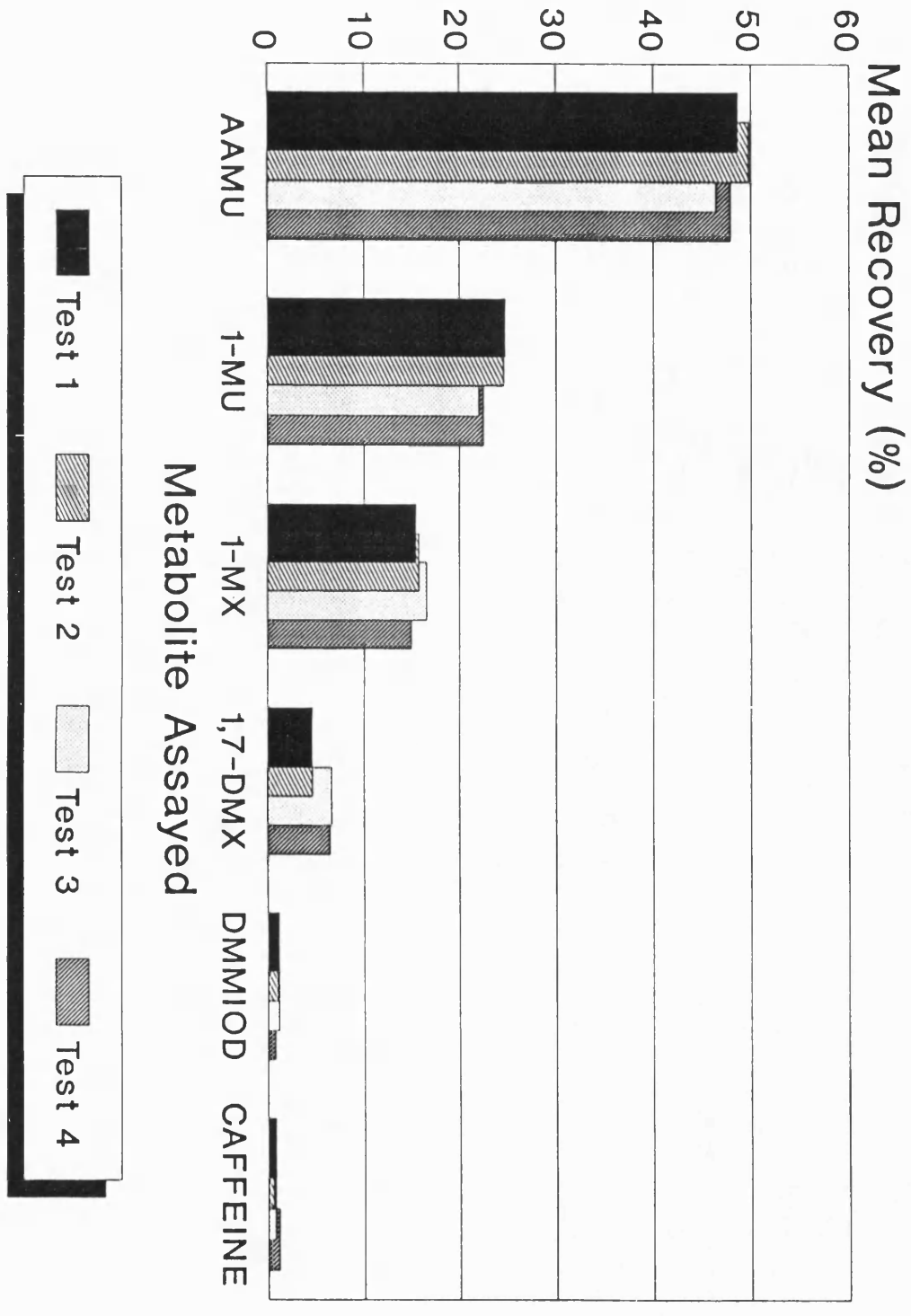


Fig.4.1.1 - Recovery (%) of caffeine metabolites in urine.

Table 4.1.2 - Total mgs of caffeine metabolites in 0-8 h urine samples following consumption of a single CCB (n=10)

Metabolite	Total mg (mean ± SEM)	Range	% Change in mean compared to first test
AAMU	33.71 ± 9.21	13.9 - 99.7	13.8
1-MU	16.67 ± 2.99	9.0 - 38.4	17.5
1-MX	10.66 ± 1.92	3.9 - 23.6	10.6
1,7-DMX	3.15 ± 0.43	1.4 - 5.8	15.6
*DMMIOD	0.70 ± 0.10	0.3 - 1.3	27.1
Caffeine	0.36 ± 0.09	0.1 - 1.05	72.2

* Originally thought to be the caffeine metabolite 1,7-DMU.

Reproducibilty of this caffeine test for each of the 10 volunteers was determined by comparing the metabolite ratios (MR) used to measure P450IA, NAT and XO activities, on study days 2-3 months apart. P450IA activity ($r=0.7333$, $p<0.05$) (Fig.4.1.2A) and NAT activity ($r=0.7173$, $p<0.05$) (Fig.4.1.2B) were not significantly different when measured on study days 2-3 months apart, while XO activity ($r=0.6485$, $p=0.0517$) (Fig.4.1.2C) tended to correlate on different test days.

4.1.3 Metabolite profile in 8 h urine, following consumption of several CCBs (Test 3).

Nineteen of the 23 healthy subjects who participated in previous protocols were studied. They had a mean age of 32.95 ± 2.15 y

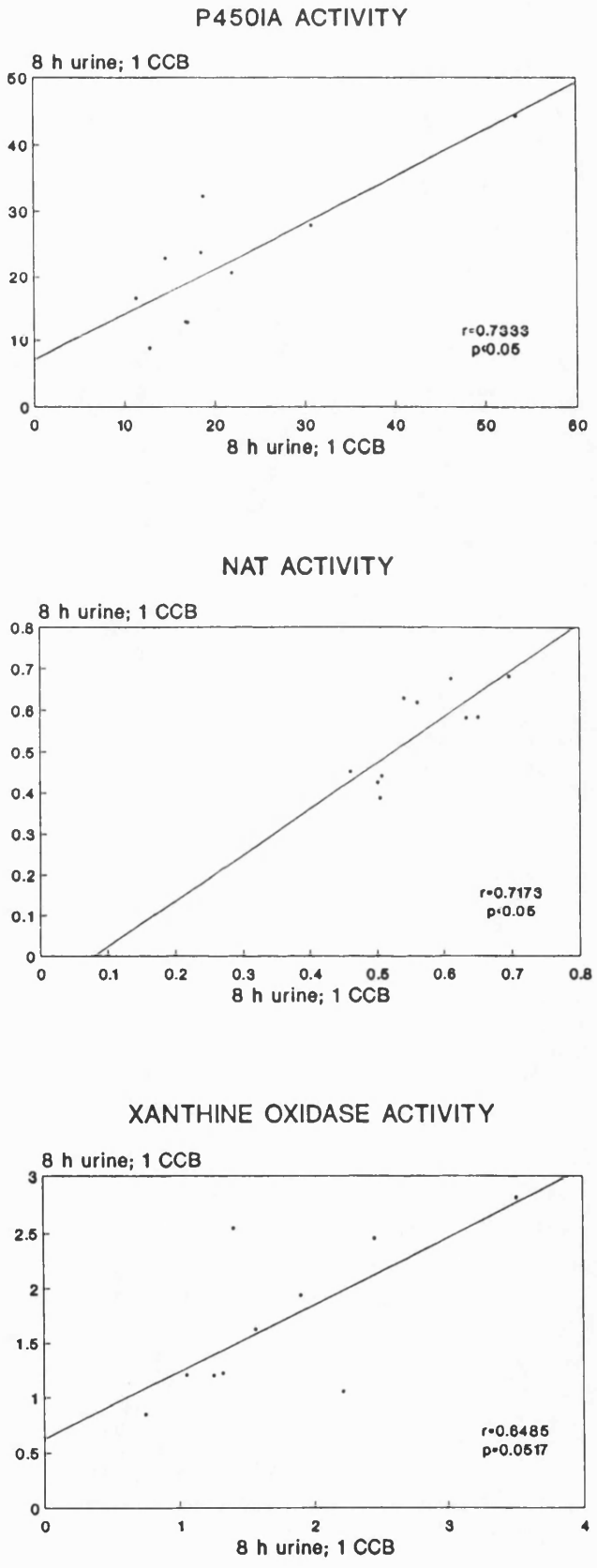


Figure 4.1.2 - Reproducibility of A) P450IA, B) NAT and C) Xanthine oxidase activity in 10 volunteers.

and consumed CCBs at breakfast, coffee, lunch and tea time throughout the working day until four CCBs in total had been consumed. Total amount (mg) of each metabolite measured is shown in Table 4.1.3 (Appendix 1c).

Table 4.1.3 - Recovery of caffeine metabolites in 0-8 h urine samples following consumption of four CCBs each (n=19)

Metabolite	Total mg (mean ± SEM)	Range
AAMU	43.69 ± 6.59	13.1 - 107.0
1-MU	20.74 ± 2.58	6.2 - 37.8
1-MX	15.58 ± 2.29	5.2 - 40.0
1,7-DMX	6.29 ± 0.82	2.2 - 15.1
*DMMIOD	1.06 ± 0.14	0.4 - 2.61
Caffeine	0.64 ± 0.18	0.05 - 2.82

* Originally thought to be the caffeine metabolite 1,7-DMU.

The major metabolites recovered were similar to other tests (Fig.4.1.1). As in the first test, correlations were found for the % recoveries of 1,7-DMX and DMMIOD ($r=0.5116$, $p<0.05$); AAMU and 1-MX ($r=-0.6789$, $p<0.005$) and AAMU and 1-MU ($r=-0.7491$, $p<0.005$). None of the other metabolites correlated either with each other or with total urine volume.

There were significant correlations between P450IA ($n=13$, $r=0.6575$, $p<0.05$), NAT ($n=13$, $r=0.5502$, $p=0.0567$) and XO ($n=13$,

r=0.8736, $p<0.05$) activities in this caffeine test involving several CCBs and the first test involving consumption of a single CCB. Enzyme activities from the caffeine test involving several CCBs were also compared to the repeat test involving consumption of a single CCB. P450IA ($n=9$, $r=0.7667$, $p<0.05$), NAT ($n=9$, $r=0.8034$, $p<0.05$) and XO ($n=9$, $r=0.8$, $p<0.05$) activities were again found to correlate significantly.

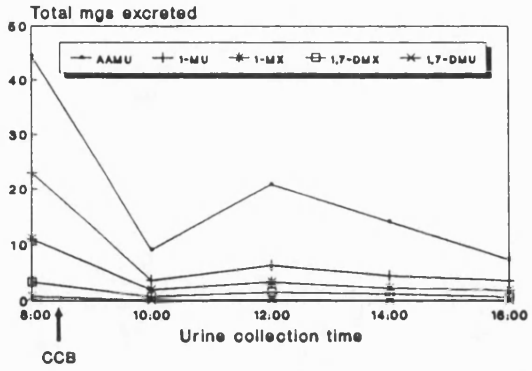
4.2 EFFECT OF URINE COLLECTION TIME ON METABOLITE PROFILES

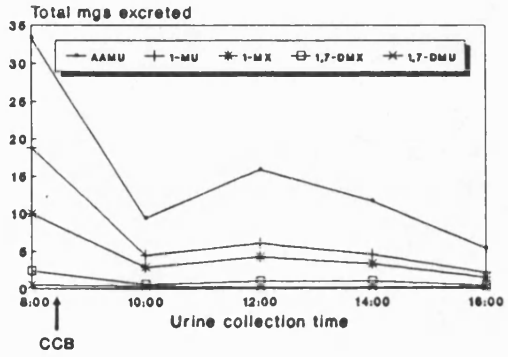
4.2.1 Urinary metabolite profile in 2 hourly samples for 8 h, following consumption of a single CCB.

In seven of the subjects who had participated in the previous tests, urine samples were collected immediately following consumption of a single CCB, and every 2 h over an 8 h period.
The major metabolites assayed in urine were similar to previous results. Total amount (mg) of metabolites measured for each subject were compared (Table 4.2.1) (Appendix 2a).
Metabolite profiles for subjects #2, #9 and #16 are shown in Figs 4.2.1A, B and C. P450IA, NAT and XO activities were also determined over 2 h periods. Results for subjects #2, #9 and #16 are shown in Figs.4.2.2A, B and C, respectively.

4.2.2 Urinary metabolite profile in 2 hourly samples for 8 h, following consumption of several CCBs

In the same seven subjects, urine samples were collected immediately following consumption of CCB at breakfast and every 2 h over an 8 h period. During this test, CCBs were also consumed at coffee, lunch and tea time throughout the working day; thus 4 CCBs were consumed in total by each subject. Total





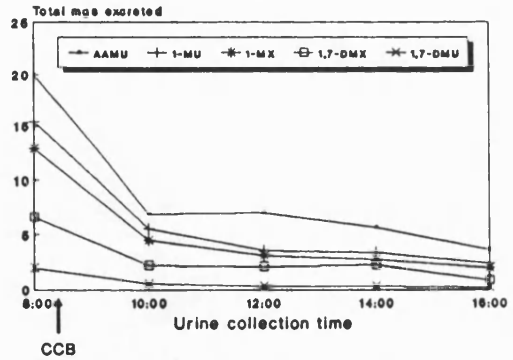
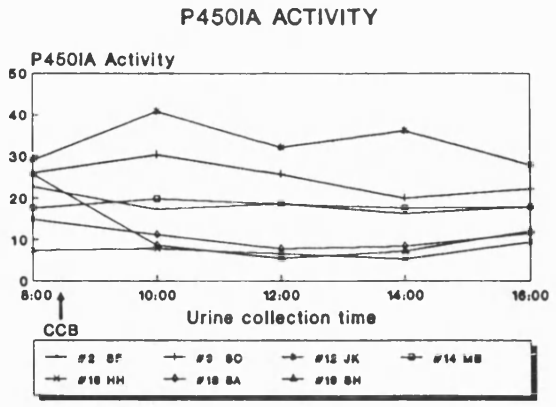
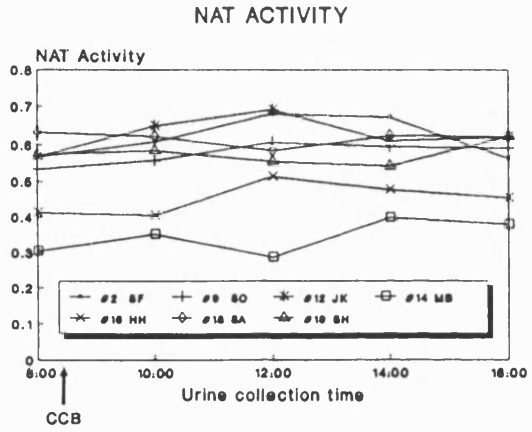


Figure 4.2.1 -Urinary excretion of individual caffeine metabolites in volunteers A) #2, B) #9 and C) #16, following consumption of a single CCB.





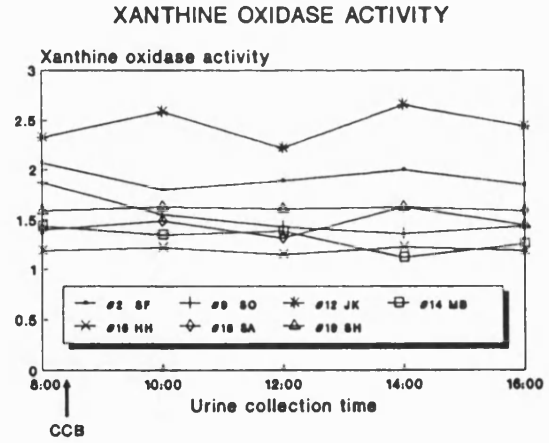


Figure 4.2.2 - A) P450IA, B) NAT and C) Xanthine oxidase activity in 7 volunteers, following consumption of a single CCB.

Table 4.2.1 - Total mgs of caffeine metabolites in urine sampled 2 hourly for 8 h, following consumption of a single CCB (n=7)

	Total mg (mean ± SEM)				
Vol #	AAMU	1-MU	1-MX	1,7-DMX	*DMMIOD
2 SF	15.4 ±6.8	6.2 ±3.7	3.3 ±1.7	1.4 ±0.5	0.21 ±0.1
9 SO	12.6 ±4.9	5.5 ±3.0	3.6 ±1.5	0.9 ±0.4	0.23 ±0.1
12 JK	15.3 ±5.0	6.4 ±2.9	2.6 ±1.3	0.7 ±0.3	0.12 ±0.1
14 MB	12.1 ±7.8	13.1 ±11	10.0 ±7.5	1.9 ±1.5	0.17 ±0.1
16 HH	7.2 ±2.9	4.8 ±2.4	3.9 ±2.0	2.3 ±1.0	0.50 ±0.3
18 SA	24.2 ±15.2	8.9 ±5.0	6.0 ±3.6	3.8 ±1.4	0.40 ±0.2
19 SH	14.4 ±4.2	6.5 ±1.7	4.8 ±0.9	2.5 ±1.0	0.18 ±0.1

* Originally thought to be the caffeine metabolite 1,7-DMU.

amount (mg) of metabolites measured for each subject are shown in Table 4.2.2 (Appendix 2b). P450IA, NAT and XO activities for each subject were also determined over 2 h periods and the results for subjects #2, #9, and #16 shown in Figs.4.2.3A, B and C, respectively.

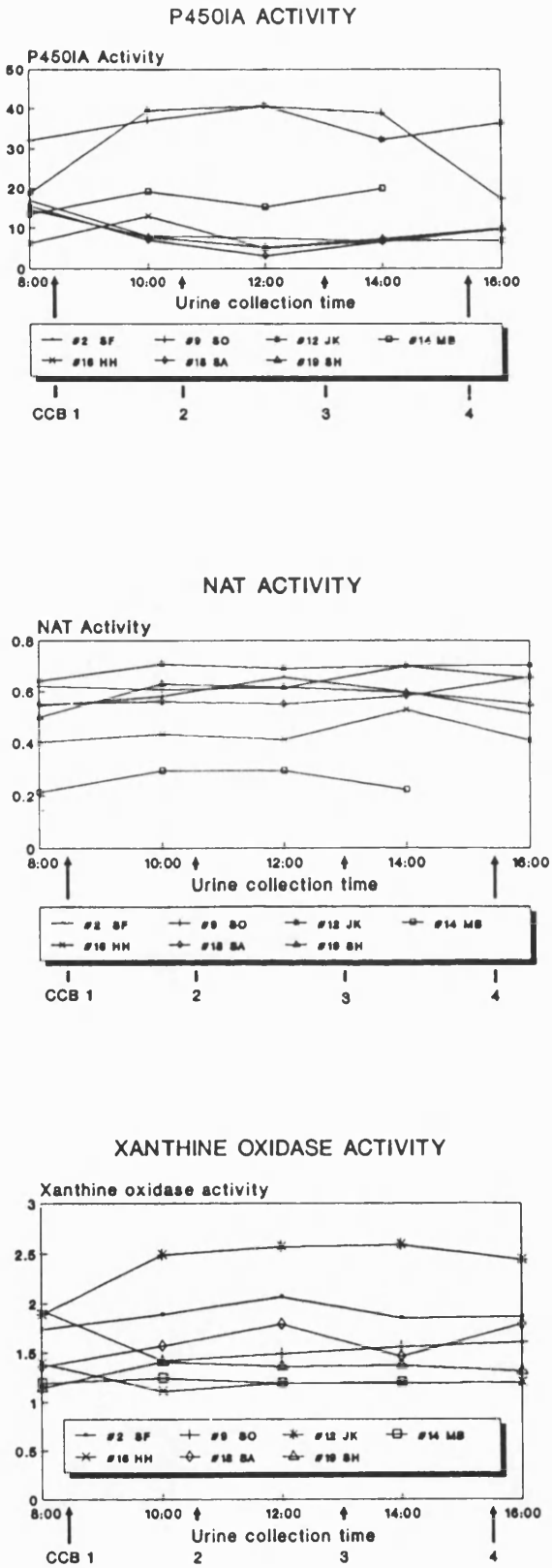


Figure 4.2.3 - A) P450IA, B) NAT and C) Xanthine oxidase activity in 7 volunteers, following consumption of four CCBs.

Table 4.2.2 - Total mgs of caffeine metabolites in urine sampled 2 hourly for 8 h, following consumption of four CCBs (n=7)

Vol #		Total mg (mean ± SEM) AAMU	1-MU	1-MX	1,7-DMX	*DMMIOD
2	SF	15.7 ±8.2	7.5 ±4.5	3.9 ±2.6	2.9 ±0.7	0.47 ±0.1
9	SO	17.8 ±11.7	6.1 ±4.9	3.8 ±2.4	0.9 ±0.6	0.19 ±0.2
12	JK	26.0 ±9.3	8.2 ±3.4	3.4 ±1.8	1.2 ±0.8	0.26 ±0.1
14	MB	10.3 ±5.0	16.4 ±11	13.6 ±9.3	2.4 ±2.0	0.49 ±0.2
16	HH	4.9 ±1.3	3.5 ±1.3	2.9 ±0.9	1.6 ±0.6	0.45 ±0.2
18	SA	15.5 ±2.8	6.8 ±1.4	4.3 ±1.1	3.6 ±0.6	0.27 ±0.03
19	SH	19.7 ±5.1	8.1 ±1.5	6.2 ±1.0	4.1 ±1.6	0.45 ±0.1

* Originally thought to be the caffeine metabolite 1,7-DMU.

4.2.3 Metabolite profile in spot urine samples collected 2-6 h afer CCB consumption (Test 4).

Seventeen healthy subjects who participated in previous protocols were studied. They had a mean age of 32.24 ± 1.84 y. Spot urine samples were collected 2-6 h after CCB consumption. Total amount (mg) of each metabolite measured is shown in Table 4.2.3 (Appendix 1d).

The major metabolites recovered are compared in Fig.4.1.1. As for previous caffeine test, correlations were found for the % recoveries of AAMU and 1-MX ($r=-0.7431$, $p<0.005$) and AAMU and 1-MU ($r=-0.7713$, $p<0.005$). In this test, % recoveries of 1,7-DMX and DMMIOD were also related ($r=0.5101$, $p<0.05$).

Table 4.2.3 - Total mgs of caffeine metabolites in spot urine samples collected 2-6 h after consumption of a CCB (n=17).

Metabolite	Total mg (mean ± SEM)	Range
AAMU	13.79 ± 3.29	2.4 - 55.6
1-MU	6.29 ± 1.00	1.9 - 16.4
1-MX	4.18 ± 0.70	1.4 - 12.7
1,7-DMX	1.81 ± 0.24	0.4 - 4.00
*DMMIOD	0.23 ± 0.05	0.05 - 0.76
TMX	0.35 ± 0.07	0.03 - 1.03

* Originally thought to be the caffeine metabolite 1,7-DMU.

There were significant correlations between P450IA ($r=0.7868$, $p<0.005$), NAT ($r=0.7448$, $p<0.005$) and XO ($r=0.6687$, $p<0.01$) activities in this caffeine test involving spot urine sample collection 2-6 h after a CCB, and the test involving 0-8 h urine collection after four CCBs.

4.3 Discussion

While caffeine is considered to be a good probe drug for estimating the activities of several enzymes, there are as yet no prototype methods in the literature which are routinely followed. Some groups have collected pooled urine samples following caffeine consumption (Grant et al, 1983a; Campbell et al, 1987; Carbo et al, 1989; Jonkman et al, 1991) while others

have collected spot samples (Grant et al, 1984; Hardy et al, 1988; Kilbane et al, 1990; Vineis et al, 1990; Bartsch et al, 1990) or first-void (overnight) urine samples (Tang et al, 1987). Rather than follow one of the published methods, several studies were undertaken to investigate the effects of varying effective caffeine dose and urine collection times on caffeine metabolism, to determine as simple a test as possible.

Initially, pooled 0-8 h urine samples were collected following consumption of a single CCB in 17 volunteers. Analysis of urine revealed AAMU, 1-MU, 1-MX and 1,7-DMX as the principal metabolites of caffeine, in agreement with previous studies (Cornish & Christman, 1957; Aldridge et al, 1979; Wietholtz et al, 1981; Kotake et al, 1982; Bonati et al, 1982; Callahan et al, 1982; Grant et al, 1983b; Campbell et al, 1987) indicating that the initial 3-demethylation of caffeine to paraxanthine is the predominant pathway in man. This pattern was followed for all other caffeine tests performed.

Before AAMU and AFMU were found to be major metabolites of caffeine by Callahan et al in 1982, earlier studies did not assay acetyluracil metabolites and therefore found 1-MU to be the major metabolite excreted in urine. In addition, Grant et al (1983b) and Campbell et al (1987) did not measure total acetyluracil but AFMU alone and found that AFMU was excreted in lower amounts than 1-MU, 1-MX, 1,7-DMU and 1,7-DMX. This may explain why percentage recoveries of caffeine metabolites were so low in these studies. Callahan et al (1982, 1983) have shown that formation of polar ring-opened uracil metabolites of paraxanthine may account for up to 40% of caffeine metabolism in

man, which is more consistent with the present data; these may also be explained by the fact that a sensitive assay for AAMU was developed. AFMU in urine was deliberately deformylated to stable AAMU so that total acetyluracil formed from paraxanthine could be assayed. Differences in measurements are almost undoubtedly due to the differences in methodology used.

In the present study, although the quantities of each metabolite were found to vary markedly between subjects, the total amount (mg) excreted by each individual was not significantly different when the 0-8 h caffeine test was repeated 2 - 3 months later. When the amounts of each metabolite were expressed as a percentage of the total metabolites measured (designated recovery %) (Fig.4.1.1), the variation between subjects was less and not significantly different over a 2-3 month period. The 0-8 h test following consumption of a single CCB was therefore reproducible and P450IA, NAT and XO activities were not significantly different on different study days.

During the first tests it proved difficult to restrict volunteers from consuming further CCBs over an 8 h period - a problem not encountered with most test drugs. It was therefore desirable to measure caffeine and its metabolites without restricting caffeine consumption. To test the effect of increasing the caffeine dose on metabolite profiles, 19 healthy volunteers participated in a different caffeine test and consumed CCBs at breakfast, coffee, lunch and tea time throughout the working day, until four CCBs in total, had been consumed. 0-8 h urine samples were again collected and pooled. Total amounts (mg) of each metabolite excreted during this study

were higher than in either test involving collection of 8 h urine after a single CCB; but not significantly so. This may be explained by the fact that although the number of drinks of coffee and tea consumed has often been used as an index of caffeine intake (Curatolo & Robertson, 1983; Barone & Roberts, 1984) this number is in fact a poor index of actual caffeine intake, due to very large differences in caffeine content and volume per drink (Lelo et al, 1986c). Furthermore, caffeine intake itself correlates poorly with 24 h average caffeine concentration in vivo (Lelo et al, 1986c). Percentage recoveries of each metabolite were not significantly different between the single- and multi- dose caffeine tests in the present studies. In addition, P450IA, NAT and XO activities were not significantly altered by the amount of caffeine consumption. These results suggest that caffeine MRs were unaffected by increasing the amounts of caffeine consumed throughout the test and indicated that restrictions on caffeine consumption were not necessary.

Campbell et al (1987) found that their caffeine metabolite ratios, determined under a regimen of multiple caffeine administrations were similar to the ratios obtained using a pooled 24 hour urine sample after ingestion of a single caffeine dose. Similar results have been found in other studies (Grant et al, 1984; Tang et al, 1987) comparing single and multi- caffeine doses. It might have been expected that increasing the caffeine dose would not alter its metabolite profiles as Bonati et al (1982) reported that caffeine was eliminated by first-order kinetics and that its metabolism was dose independent at the doses of caffeine to which man is normally exposed, in agreement

with Parsons & Neims (1978). Further studies supported this and reported that caffeine followed linear kinetics up to 10 mg/kg in man (Blanchard & Sawers, 1983; Newton et al, 1981). Also, theobromine disposition was found to be unaffected when subjects consumed theobromine (6 mg/kg/day) for 7 days in the form of dark chocolate (Shively et al, 1985).

Initial caffeine tests performed on the general population therefore involved collection of 0-8 h urine samples following consumption of a CCB, without restriction on further CCB consumption.

For widepread use of the caffeine test, it was desirable that spot urine samples could be collected. It was also important to try and shorten the urine collection period for the patient studies, most of whom were attending outpatient clinics. In order to investigate the effects of varying urine collection times, 7 subjects who had participated in all of the caffeine tests provided 2 hourly urine samples over an 8 h period following consumption of either a single or several CCBs.

Although the total amount (mg) of each metabolite excreted for each volunteer were higher in the urine samples after 4 CCBs than those following a single CCB, the results were not significantly different between the two tests, which is in agreement with our results from tests involving 0-8 h urine samples, after either single or multi- caffeine doses.

When the metabolite profiles for three of the subjects were studied after a single CCB and compared with those after several CCBs, it could be seen that for all the metabolites, very large quantities were excreted in the first-void urine samples before

caffeine had been consumed. The amounts excreted in this "blank" urine were not comparable with total amount (mg) excreted in the 2-8 h spot samples. This appeared to the case particularly for AAMU. In fact, the enzyme activities of interest, such as P450IA, NAT and XO when measured in first-void samples were not as consistent as in 2-6 h spot samples, whether single or multiple CCBs were consumed. Overall, the mean amount of metabolites excreted and the enzyme activities for each volunteer were not significantly different between the two tests.

These results suggested that total amount of caffeine metabolites excreted and P450IA, NAT and XO activities for an individual were most consistent in the spot urine samples collected 2-6 hours after a CCB, which has been found by other workers (Grant et al, 1984) who required a simplified caffeine test for routine use. Indeed, MRs for the test involving collection of a spot urine sample 2-6 h after a single CCB correlated well with those determined in the tests involving collection of 0-8 h urine. In addition, Campbell et al (1987) showed that randomly collected urine samples after chronic dietary caffeine intake were adequate for the assessment of their caffeine MRs in population studies, particularly when routine dietary intake patterns approached steady-state conditions. The present study did not support the idea that first-void urine samples provide the best method for determining caffeine MRs because even if the MRs were highly reproducible in these urine samples, as reported by Tang and coworkers (1987), they did not give a good representation of MRs determined at other times throughout the day. It has been suggested that

trough plasma caffeine concentrations measured in the morning might give an estimate of caffeine clearance and thus liver function (Renner et al, 1984) - however, Lelo and workers (1986c) also found that the morning concentration of caffeine correlated very poorly with mean 24 h caffeine concentration, whereas plasma concentrations measured later in the day correlated well.

When metabolite profiles were studied for each caffeine test, recovery (%) of AAMU was found to correlate negatively with 1-MX ($r=-0.777$, $p<0.005$) and 1-MU ($r=-0.652$, $p<0.01$). This would be expected as the formation of these metabolites from some unknown, unstable ring-opened intermediate depends on the acetylation status of an individual. In a rapid acetylator, more of this intermediate will be acetylated to form AAMU, whereas in a slow acetylator, more of the intermediate will reclose to form 1-MX and therefore in turn, 1-MU. The negative correlations found in this study lend further support for this theory.

Recovery (%) of 1,7-DMX was found to correlate positively with amounts of DMMIOD excreted in urine ($r=0.7212$, $p<0.05$). Unextracted authentic 1,7-DMU standard had a very similar retention time to DMMIOD. Subsequent studies found that authentic 1,7-DMU could not be extracted in chloroform and isopropanol mixes, unlike other dimethylxanthine and uric acid products of caffeine. This is very unusual in light of other groups that have supposedly extracted 1,7-DMU (Adams Chamicals, USA) in these organic solvents (Grant et al, 1983a). It is of interest however that the DMMIOD chemical originally measured in urine could be extracted with other caffeine metabolites, eluted very closely to 1,7-DMX and was present in every urine sample

analysed. It was almost certainly a caffeine related compound, as it was absent from the one subject who abstained form CCBs for 72 hours in order to obtain a blank urine sample. Amounts of DMMIOD excreted in urine seemed to parallel amounts of 1,7-DMX but were not present when 1,7-DMX standard was extracted alone. It was unlikely therefore to be a contaminant or by-product of another standard. Present knowledge of this compound is that it has never been described as a caffeine metabolite. These results indicate that this may be the case and warrant further investigation.

In summary, the method involving collection of spot urine samples is suitable for use in outpatient clinics, requiring only that patients consume a CCB 2-6 h prior to urine collection. The urinary test is suitable for large-scale population studies because it requires only dietary intake of caffeine - a popular and relatively safe substance - and because it relies on simple urine collection rather than on multiple blood sampling. In addition, this urine test is inexpensive compared to the caffeine breath test, involving use of isotopically labelled caffeine (Wietholtz et al, 1981; Kotake et al, 1982) and also has an advantage in that several enzyme activities, including P450IA, XO and NAT can be measured from the same urine sample.

CHAPTER FIVE

CYTOCHROME P450IA AND P450IA2 ACTIVITIES IN HUMAN VOLUNTEERS

5.1 P450IA AND P450IA2 ACTIVITIES IN HEALTHY INDIVIDUALS

5.1.1 Introduction

Variation in P450IA1 (Pelkonen *et al*, 1979) and P450IA2 (Wrighton *et al*, 1986) activities have been reported in man. The cause of such variation may be attributed to several factors. Firstly, the P450IA isozymes are inducible, such that individuals exposed to certain environmental chemicals, such as components of cigarette smoke, may have a higher enzyme activity than their basal levels. Also, P450 enzyme activities can be inhibited by several drugs, including oral contraceptive steroids (OCS). In addition, several P450 enzymes, including possibly the P450IA isozymes, are subject to genetic polymorphism. Such inherited variation in enzyme activity may result in large differences in drug biotransformation.

By giving caffeine to a large number of control volunteers and measuring caffeine metabolite ratios, the effects of both environmental and genetic factors on caffeine N-demethylation were examined.

5.1.2 Results

The 127 subjects who participated in the caffeine test involving collection of 0-8 h urine (Appendix 3) had a mean P450IA activity (MR) of 13.86 $\pm$ 1.44. 105 of these subjects were also studied for P450IA2 activity. Metabolite ratios were not measured in 22 volunteers as the amount of caffeine was not analysed. The mean P450IA2 activity was 96.64 $\pm$ 8.73.

For the 150 volunteers who participated in the caffeine test involving collection of a spot urine sample 2-6 h after a CCB (Appendix 4), the mean P450IA activity was 15.07 ± 1.04. 149 of these subjects were also studied for P450IA2 activity. The metabolite ratio was not measured in one of these subjects as the amount of caffeine was not analysed. Mean P450IA2 activity was 97.70 ± 6.99. P450IA and P450IA2 activities in the 2-6 h caffeine test were not significantly different from those in the 0-8 h test ($p>0.05$), in agreement with previous results (Section 4.2).

In light of the fact that neither the amount of caffeine consumption (Section 4.1) or the time of urine collection (Section 4.2) altered caffeine metabolite ratios, the results from the 0-8 h and the 2-6 h tests were combined.

Table 5.1.1 - P450IA and P450IA2 activities determined in the caffeine tests

Enzyme	n	Median	Mean activity ±SEM	Range
P450IA	277	11.64	14.52 ± 0.87	1.15 - 130.33
P450IA2	254	74.68	97.29 ± 5.45	7.92 - 607.62

Mean P450IA and P450IA2 activities determined for all subjects are shown in Table 5.1.1. For the each volunteer who had both P450IA and P450IA2 activities determined, correlations between the two enzymes were examined. The enzyme activities were highly significantly correlated ($n=254$; $r=0.4228$; $p<0.00001$).

5.1.3 Effect of Age

277 subjects with recorded dates of birth were studied. Their age ranged between 17 and 91 y (40.42 ± 0.96 y; mean ± SEM). There was no significant difference in the age of the subjects participating in the 0-8 h or the 2-6 h caffeine tests (40.67 ± 1.19 y vs 42.83 ± 1.49 y; $p>0.1$). In addition, there was no significant difference between the age of the males (n=145) and females (n=132) participating in the caffeine tests (40.06 ± 1.27 y vs 40.74 ± 1.46 y; $p>0.5$). No correlation was found between subject age and the degree of either P450IA ($r=-0.0846$, $p>0.5$) or P450IA2 activity ($r=-0.1234$; $p>0.05$).

5.1.4 Effect of Body Mass Index

The effect of body mass index (BMI) was studied in 254 subjects. Twenty-three without recorded heights (m) and weights (kg) were excluded from this study.

BMI = body weight (kg)/ height in metres squared.

129 females had BMI values ranging between 17.40 - 52.14 (23.72 ± 0.37; mean ± SEM). 20 of these subjects had values outside the normal range (>26.7). 125 males had BMI values ranging between 17.04 - 34.33 (24.45 ± 0.26) and 22 of these subjects had values >26.7. BMI was not significantly different between males and females ($p>0.1$). No correlation was found between BMI values and the activity of P450IA ($r=-0.0732$; $p>0.5$) or P450IA2 ($r=0.0053$; $p>0.5$).

5.1.5 Effect of Subject Gender

In order to compare the sexes, values for P450IA and P450IA2 activities were logged and results compared by Student's t-test.

P450IA activity was determined in 145 males and 132 females. Of the females, 90 were non-OCS users, while 42 had been prescribed OCS or hormone replacement therapy (HRT).

P450IA activity was not significantly different between males and females (Table 5.1.2). This relationship was obtained whether males were compared with non-OCS user females (n=90; $p>0.1$) or the whole female population, including OCS and non-OCS users (n=132; $p>0.1$).

Table 5.1.2 - P450IA and P450IA2 activities in male and female populations

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Males	145	11.76	15.82 ± 1.37	3.06 -130.33
	Females				
	(All)	132	11.33	13.11 ± 1.02	1.15 -118.46
	(Non-OCS)	90	11.47	13.26 ± 1.38	2.95 -118.46
P450IA2	Males	125	74.67	94.15 ± 6.23	8.56 -395.22
	Females				
	(All)	129	74.69	100.34 ± 8.89	7.92 -607.62
	(Non-OCS)	87	78.04	109.58 ±12.02	11.58 -607.62

P450IA2 activity was determined in 125 males and 129 females. Of the females, 87 were non-OCS/HRT users and 42 were taking OCS or HRT. P450IA2 activity was also found to be not significantly different between the male and female populations (Table 5.1.2).

This was the case whether males were compared to non-OCS user females (n=87; p>0.5) or the whole female population (n=129; p>0.1).

5.1.6 Effect of Oral Contraceptive Steroids

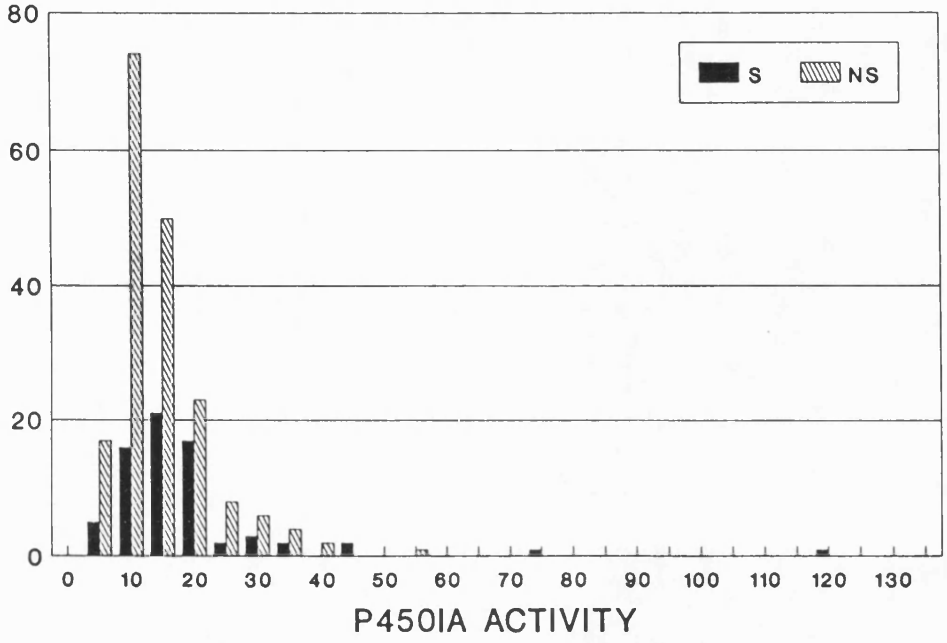
P450IA activities (Table 5.1.3) in women using OCS/HRT were not significantly different from those measured in women not taking steroids (p>0.5). P450IA2 activities (Table 5.1.3) were also not significantly altered by OCS/HRT use (p>0.1).

Table 5.1.3 - P450IA and P450IA2 activities in OCS and non-OCS using females

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	OCS	42	10.67	12.80 ±1.25	1.15 -40.35
	Non-OCS	90	11.47	13.25 ±1.38	2.95 -118.46
P450IA2	OCS	42	54.50	80.95 ±10.84	7.92 -292.22
	Non-OCS	87	78.04	109.58 ±12.02	11.58 -607.62

5.1.7 Effect of Cigarette Smoke

P450IA activity was studied in 255 individuals of known smoking status; 70 subjects were smokers and 185 were nonsmokers. P450IA2 activity was determined in 254 of these subjects; 70 were smokers and 184 were nonsmokers (Table 5.1.4). P450IA (Fig.5.1.1A) and P450IA2 (Fig.5.1.1B) activities were found to be significantly higher in smokers than nonsmokers (p<0.05).



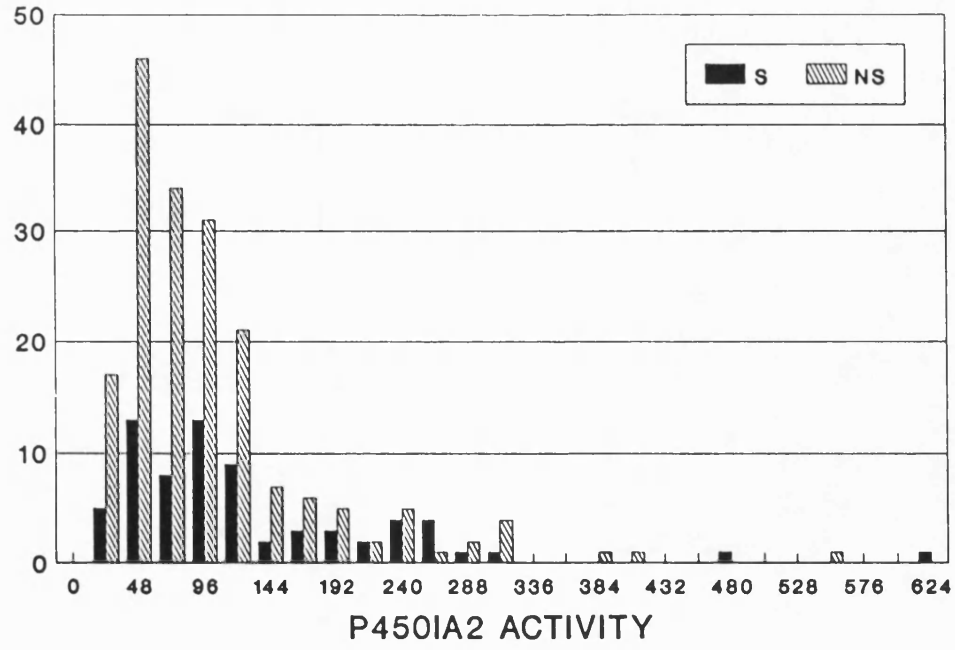


Figure 5.1.1 - The effect of cigarette smoking on A) P450IA and B) P450IA2 activities.

Table 5.1.4 - P450IA and P450IA2 activities in smoking and nonsmoking healthy populations

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Smokers	70	13.76	16.55 ± 1.94	1.15 -118.46
	Nonsmokers	185	10.09	12.21 ± 0.57	2.95 - 54.89
P450IA2	Smokers	70	85.48	118.66 ±12.49	7.92 -607.62
	Nonsmokers	184	68.90	89.49 ± 5.74	1.58 -550.23

Subpopulations of smokers and nonsmokers were also studied, to determine the effect of cigarette smoking on P450IA (Table 5.1.5) and P450IA2 (Table 5.1.6) activities of groups with different gender and OCS/HRT use.

When the nonsmoking populations were studied, P450IA and P450IA2 activities were not significantly different between males and females, whether they were taking OCS/HRT or not ($p>0.1$). In addition, P450IA and P450IA2 activities were not significantly different between male and female smokers ($p>0.1$), though results show that the difference in enzyme activities between smokers and nonsmokers was greater in females than in males.

In the female population who were not taking OCS/HRT, the smokers had a significantly higher P450IA ($p<0.0005$) and P450IA2 activity ($p=0.05$) than the nonsmokers. However, when the women using OCS/HRT were studied, there was no significant difference in P450IA ($p>0.5$) or P450IA2 ($p>0.1$) between smokers and nonsmokers. Similarly, when the male population was analysed,

Table 5.1.5 - P450IA activity in smoking and nonsmoking male and female populations

Group	n	Median	Mean activity ± SEM	Range
Males				
-smokers	35	12.08	15.86 ±2.20	3.06 -71.62
-nonsmokers	91	10.45	12.92 ±0.88	3.23 -38.90
Females				
Non-OCS/HRT	90			
-smokers	17	15.11	21.07 ±6.12	9.43 -118.46
-nonsmokers	73	10.06	12.29 ±1.26	2.95 -79.98
OCS/HRT	42			
-smokers	18	11.24	13.62 ±2.39	1.15 -40.35
-nonsmokers	24	10.43	12.19 ±1.29	5.05 -34.82

although the smokers tended to have a higher P450IA ($p>0.5$) and P450IA2 activity ($p>0.1$) than the nonsmokers, the results were not significantly different.

In the group of 132 females, the combined effects of OCS or HRT administration and smoking status were studied (Tables 5.1.5 and 5.1.6). In the nonsmoking female population, P450IA activity was not significantly different whether females were taking OCS/HRT or not ($p>0.1$). In the smoking female population however, P450IA activity was significantly lower in females taking OCS/HRT than those who were not ($p=0.05$). Overall, in a female population of smokers and nonsmokers, P450IA activity remained unaffected by

OCS use ($p>0.5$). P450IA2 activity was not significantly different between women using OCS/HRT and those who were non-OCS users, whether the nonsmoking ($p>0.05$) or the smoking populations ($p>0.1$) were studied. Also, in the whole female population of smokers and nonsmokers, P450IA2 activity remained unaffected by OCS/HRT use ($p>0.1$). Finally, smoking women using OCS/HRT were compared to nonsmoking women who were non-OCS users, to test if OCS administration could reduce the high enzyme activities observed in female smokers. Results show this to be the case, as both P450IA and P450IA2 were not significantly different between the two groups ($p>0.5$).

Table 5.1.6 - P450IA2 activity in smoking and nonsmoking male and female populations

Group	n	Median	Mean activity ± SEM	Range
Males				
-smokers	35	86.16	112.49 ±14.49	8.56 -308.92
-nonsmokers	90	73.09	83.69 ±5.47	13.26 -242.41
Females				
Non-OCS/HRT	87			
-smokers	17	102.95	149.78 ±37.61	41.63 -607.62
-nonsmokers	70	66.30	99.82 ±11.71	11.58 -550.23
OCS/HRT	42			
-smokers	18	83.94	101.27 ±17.66	7.92 -247.74
-nonsmokers	24	43.89	66.13 ±12.99	15.72 -292.22

5.1.8 Genetic control of P450IA and P450IA2 activities

P450IA Activity

The frequency distribution histogram for P450IA activity in 277 control volunteers is depicted in Fig.5.1.2. The summary statistics for this distribution are shown in Table 5.1.7. 113-fold variation in P450IA activity existed in 277 volunteers. It can be seen from the histogram and Table 5.1.7 that the distribution is skewed.

a) The positive skewness coefficient of 5.23 shows that the distribution is strongly positively skewed. The kurtosis value of 35.66 is higher than the coefficient of zero that would be expected from a normal distribution. In addition, the standardized coefficients in Table 5.1.7 are both outside the range of -2 to +2 that would be expected from a normal distribution and therefore indicate that the data for P450IA activity may depart significantly from normality.

b) The non-linear normal probability plot (Fig.5.1.3) also indicates that the data deviates from a normal distribution.

c) This is confirmed by both the K-S test ($p<0.00001$) and Chi-square test ($p<0.00001$), which show highly significant difference from normality.

d) In addition, the Lilly-Fors test was employed to test for bimodality and deviation of the data from a normal distribution. The results for P450IA activity were significantly different ($p<0.01$) from those expected from a normal distribution.

Of the environmental factors tested, only cigarette smoking seemed to affect P450IA activity to a significant degree. Therefore, in addition to the whole population, we studied the distribution of P450IA activity separately, in smokers and

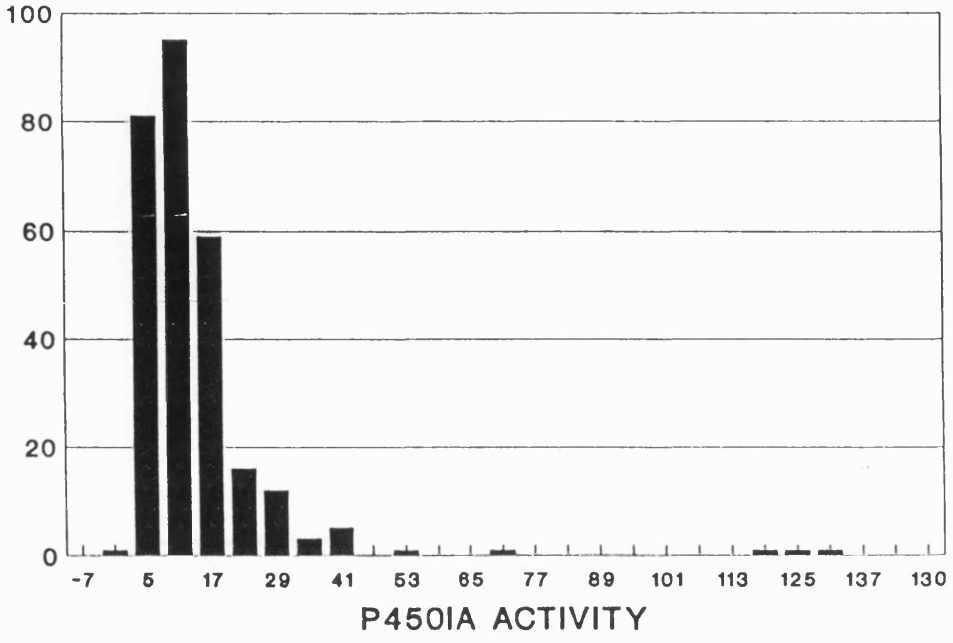


Figure 5.1.2 - Frequency distribution histogram of P450IA activity in 277 volunteers.

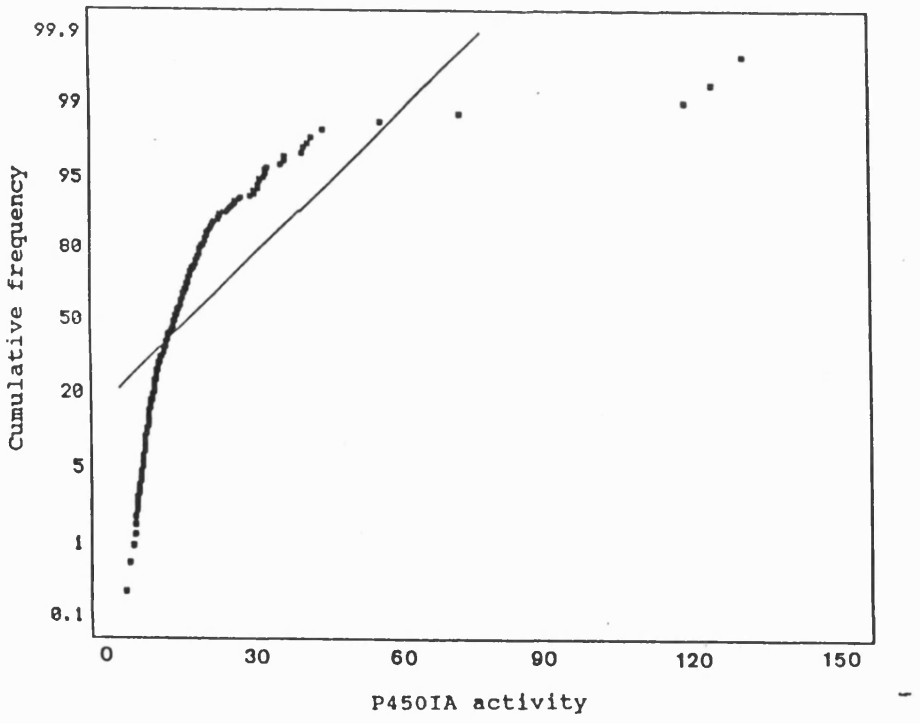


Figure 5.1.3 - Normal probability plot of P450IA activity in 277 volunteers.

Table 5.1.7 - Summary Statistics for P450IA activity in a population of 277 control volunteers

Mean	14.52
Median	11.64
Standard error	0.87
Standard deviation	14.46
Minimum	1.15
Maximum	130.33
Skewness	5.23
Standardized skewness	35.53
Kurtosis	35.66
Standardized kurtosis	121.16

nonsmokers. The histograms are shown in Figs.5.1.4 and 5.1.5, respectively and the summary statistics for these distributions are shown in Table 5.1.8. As discussed in Section 5.1.7, smokers had a significantly higher P450IA activity than nonsmokers ($p<0.05$). For the smoking population there was a 103-fold variation in P450IA activity, whereas the nonsmokers exhibited only a 19-fold difference. As seen in Table 5.1.8, the distributions of P450IA activity for both smokers and nonsmokers were positively skewed, although the smokers tended to show a less normal distribution than the nonsmokers. This could be seen more clearly when normal probability plots were compared. The plot for smokers (Fig.5.1.6) was non-linear and statistical tests confirmed that the data deviated from normal (K-S test, $p<0.0001$; Chi-square test, $p<0.00001$; Lilly-Fors test, $p<0.01$).

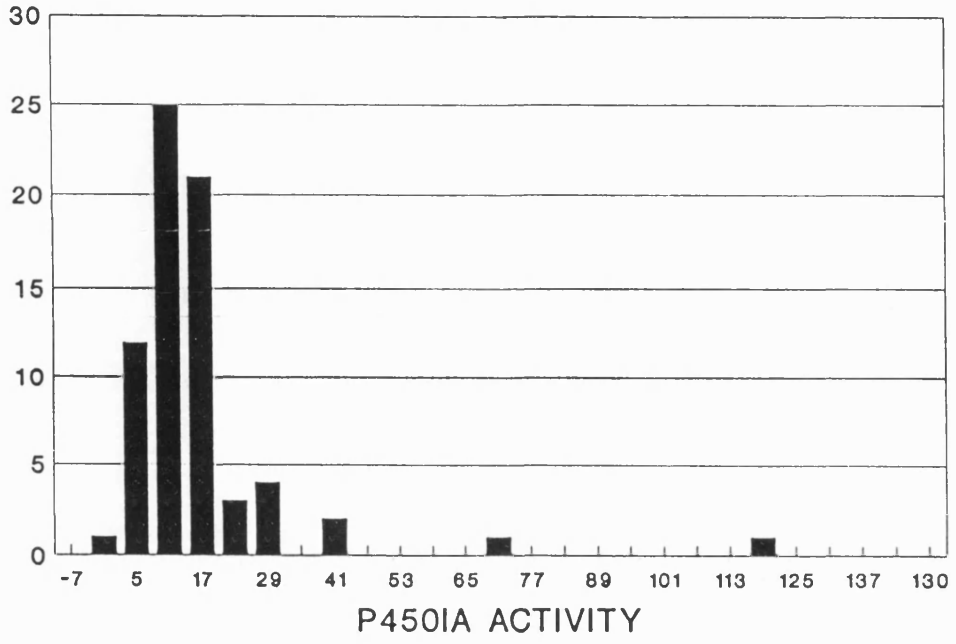


Figure 5.1.4 - Frequency distribution histogram of P450IA activity in 70 smoking volunteers.

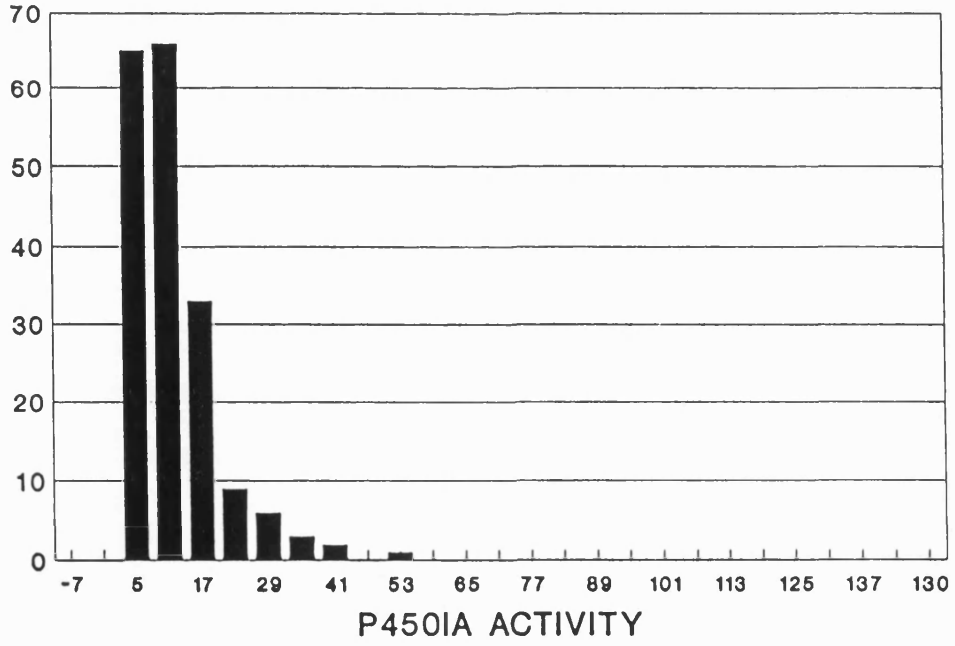


Figure 5.1.5 - Frequency distribution histogram of P450IA activity in 185 nonsmoking volunteers.

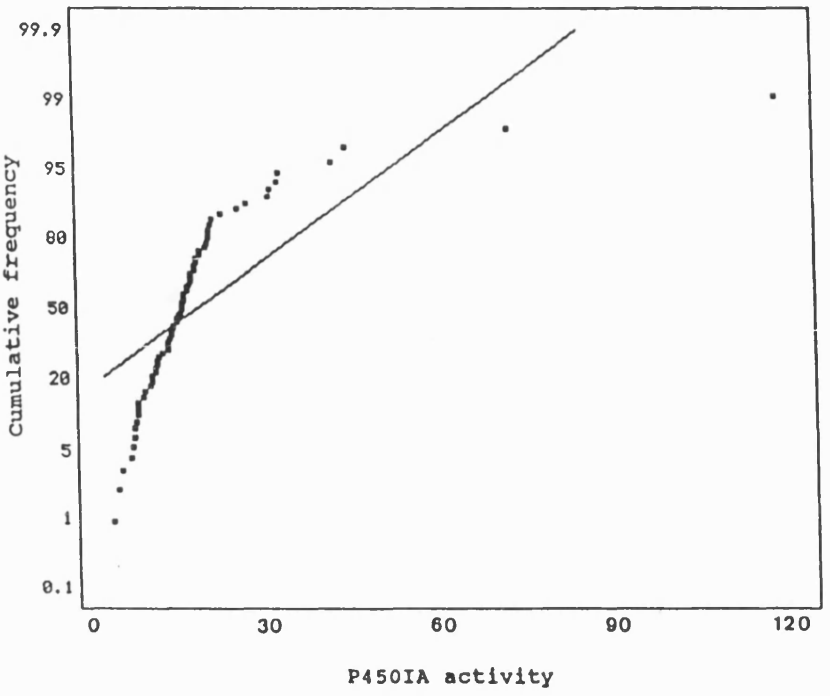


Figure 5.1.6 - Normal probability plot of P450IA activity in 70 smoking volunteers.

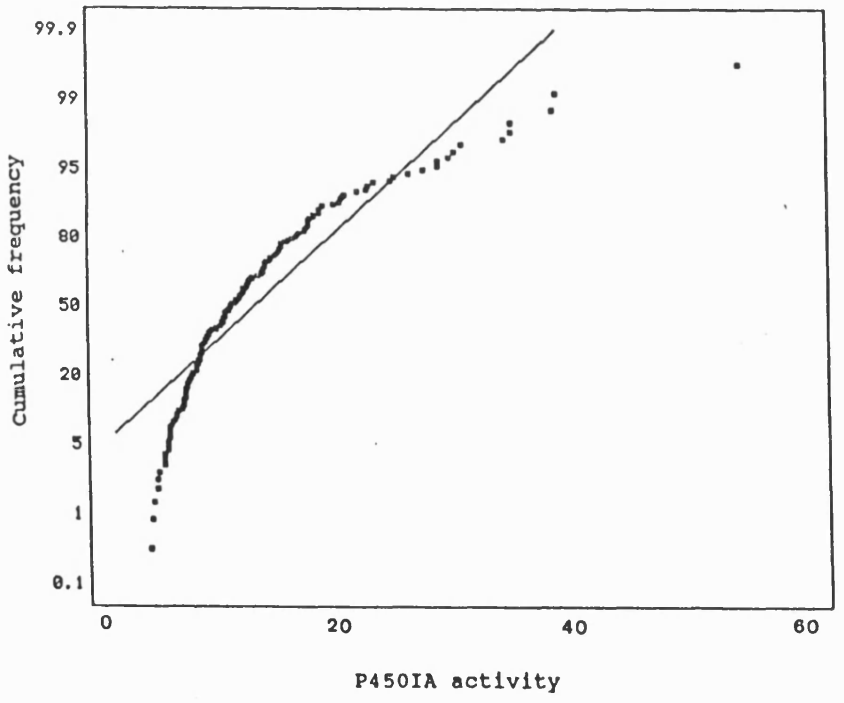


Figure 5.1.7 - Normal probability plot of P450IA activity in 185 nonsmoking volunteers.

Table 5.1.8 - Summary Statistics for P450IA activity in smoking and nonsmoking subjects

	Smokers	Nonsmokers
n	70	185
Median	13.76	10.09
Mean	16.55	12.21
Standard error	1.94	0.57
Standard deviation	16.25	7.74
Minimum	1.15	2.95
Maximum	118.46	54.89
Skewness	4.31	2.05
Standardized skewness	14.73	11.39
Kurtosis	23.67	6.06
Standardized kurtosis	40.42	16.81

Similarly, the normal probability plot for P450IA activity in nonsmokers (Fig.5.1.7) was non-linear and possibly bimodal (K-S test, $p<0.005$; Chi-square test, $p<0.00001$; Lilly-Fors test, $p<0.01$).

Due to the skewness of P450IA activity data, whether smokers or nonsmokers were considered, the data were logged and restudied in an attempt to detect bimodality of the distributions. The frequency distribution histogram for log P450IA activity in 277 control volunteers is shown in Fig.5.1.8. The summary statistics for this population are shown in Table 5.1.9.

Table 5.1.9 - Summary Statistics for log P450IA activity in a population of 277 control volunteers

Skewness	0.40
Standardized skewness	2.72
Kurtosis	1.63
Standardized kurtosis	5.53

Results in Table 5.1.9 suggest that the distribution was not normally distributed. Although both the K-S test ($p>0.4$) and the Chi-square test ($p>0.3$) results indicated that the data did not differ significantly from a normal distribution, the more sensitive Lilly-Fors test suggested that log P450IA activity was significantly different from normal and possibly bimodal ($p<0.05$).The summary statistics for log P450IA activity in smokers and nonsmokers are shown in Table 5.1.10.

Table 5.1.10 - Summary Statistics for log P450IA activity in smoking and nonsmoking subjects

	Smokers	Nonsmokers
n	70	185
Skewness	-0.22	0.25
Standardized skewness	-0.74	1.38
Kurtosis	2.40	-0.08
Standardized kurtosis	4.10	-0.22

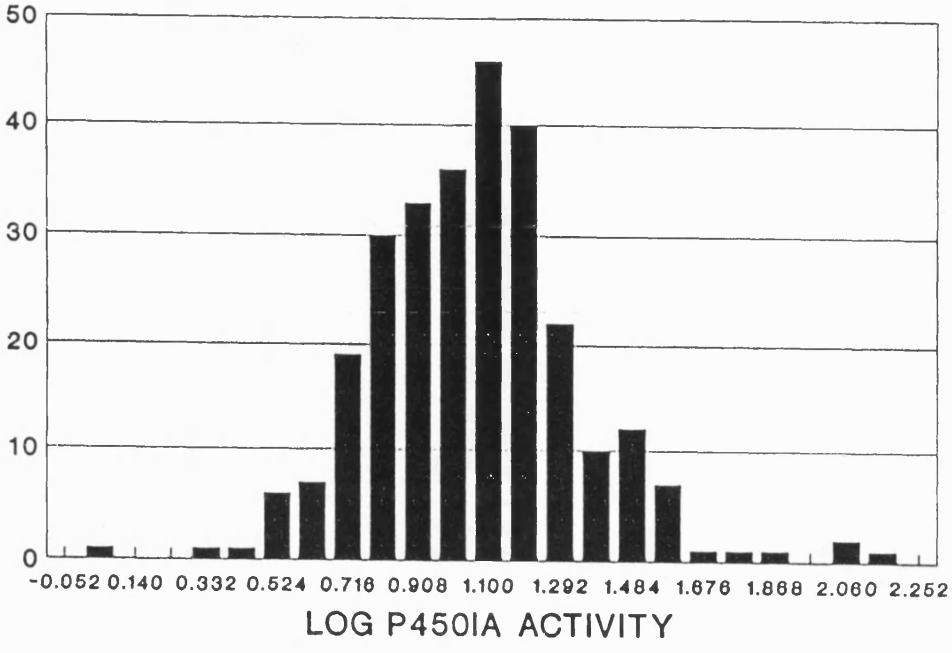


Figure 5.1.8 - Log frequency distribution histogram of P450IA activity in 277 volunteers.

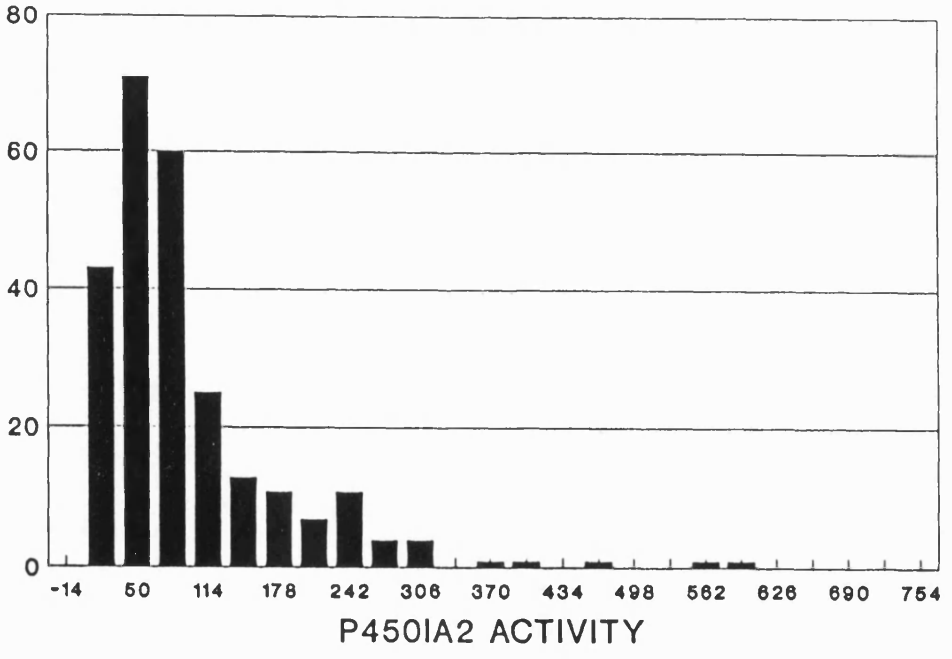


Figure 5.1.9 - Frequency distribution histogram of P450IA2 activity in 254 volunteers.

The only coefficient that differed from that of a normal distribution was the degree of kurtosis of log P450IA activity in smoking subjects. In addition, the Chi-square test showed that the data deviated from a normal distribution ($p<0.005$) and the Lilly-Fors test indicated that P450IA activity in smokers might be bimodal ($p<0.05$). Conversely, for nonsmoking subjects, it seemed unlikely that P450IA activity was bimodally distributed (K-S test, $p>0.5$; Chi-square test, $p>0.5$; Lilly-Fors test, $p>0.05$).

As the possibility of a bimodal distribution for P450IA activity cannot be rejected, a cut-off point was arbitrarily assigned in the frequency distribution to separate control volunteers in the skewed region with relatively high P450IA activity from those with enzyme activities in the normal unskewed part of the distribution. In order to achieve such separation, the apparent antimode at 26 was used (Fig.5.1.2). Any subject with a metabolite ratio greater than 26 was classified as having high P450IA activity (Table 5.1.11).

Table 5.1.11 - The proportion of control volunteers with high P450IA activity

Group	P450IA activity			
	High	%	Low	%
All	25/277	(9.0%)	252/277	(91.0%)
Smokers	8/ 70	(11.4%)	62/ 70	(88.6%)
Nonsmokers	12/185	(6.5%)	173/185	(93.5%)

9% of the 277 subjects studied were found to have high P450IA activity. 11.4% of the 70 smokers (Fig.5.1.4) and 6.5% of the 185 nonsmokers (Fig.5.1.5) were found to have high P450IA activity. The Chi-square test for comparing proportions indicated that the number of smoking and nonsmoking subjects with high P450IA activity were not significantly different ($p>0.05$).

P450IA2 Activity

The frequency distribution histogram for P450IA2 activity in 254 control volunteers is depicted in Fig.5.1.9. The summary statistics for this distribution are shown in Table 5.1.12.

Table 5.1.12 - Summary Statistics for P450IA2 activity in a population of 254 control volunteers

Median	74.68
Mean	97.29
Standard error	5.45
Standard deviation	86.88
Minimum	7.92
Maximum	607.62
Skewness	2.43
Standardized skewness	15.83
Kurtosis	8.38
Standardized kurtosis	27.26

77-fold variation in P450IA2 activity existed in 254 volunteers.

It can be seen from the histogram and Table 5.1.12 that the distribution is skewed.

a) The positive skewness coefficient of 2.43 showed that the distribution was strongly positively skewed, although not to the extent of the distribution for P450IA activity. The kurtosis value of 8.38 was also higher than the coefficient of zero that would be expected from a normal distribution. The standardized coefficients in Table 5.1.12 were both outside the range of -2 to +2 that would be expected from a normal distribution.

b) The non-linear normal probability plot (Fig.5.1.10) also indicated that the data deviates from a normal distribution.

c) This was confirmed by the K-S test ($p<0.00001$) and the Chi-square test ($p<0.00001$) results, which both showed highly significant difference from normality.

d) In addition, the Lilly-Fors test was employed to test for bimodality and deviation of the data from a normal distribution. P450IA2 activity was highly significantly different ($p<0.01$) from that expected from a normal distribution, as was the case for P450IA activity.

The frequency distributions for P450IA2 activity in smokers and nonsmokers are shown in Figs.5.1.11 and 5.1.12, respectively and the summary statistics for these distributions are shown in Table 5.1.13.

As discussed in Section 5.1.7, smokers had a significantly higher P450IA2 activity than nonsmokers ($p<0.05$). For the smoking population there was a 77-fold variation in P450IA activity, whereas the nonsmokers exhibited 48-fold difference. As seen in Table 5.1.12, the distributions of P450IA2 activity for both smokers and nonsmokers were positively skewed although

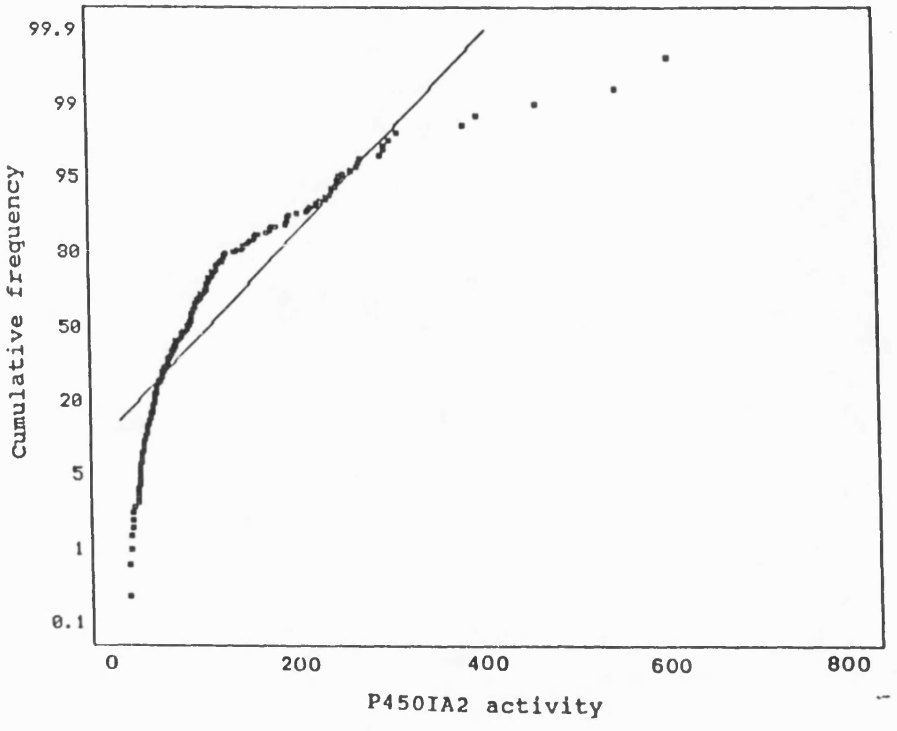


Figure 5.1.10 - Normal probability plot of P450IA2 activity in 254 volunteers.

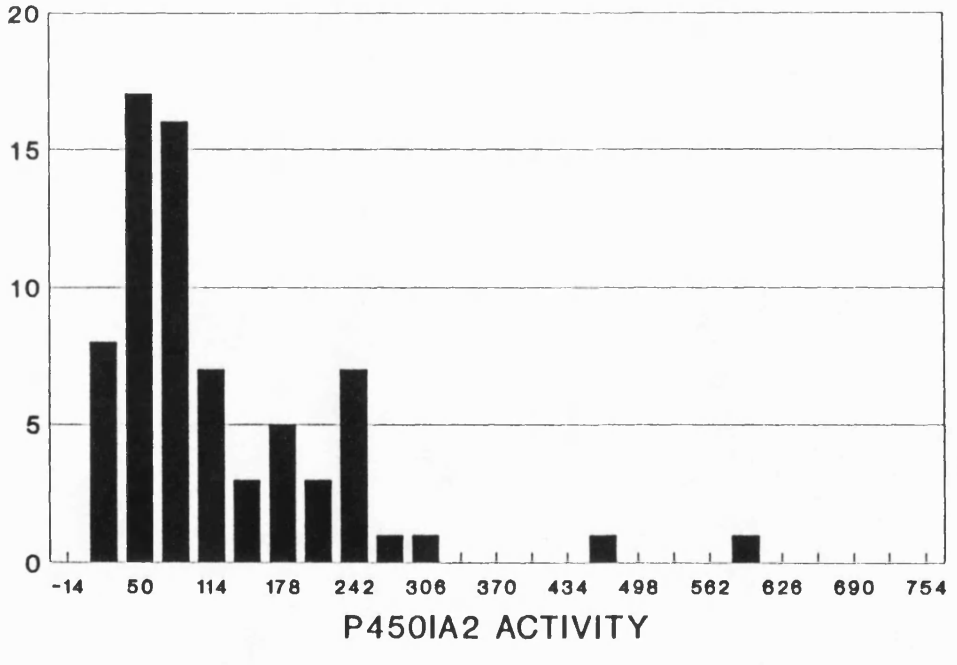


Figure 5.1.11 - Frequency distribution histogram of P450IA2 activity in 70 smoking volunteers.

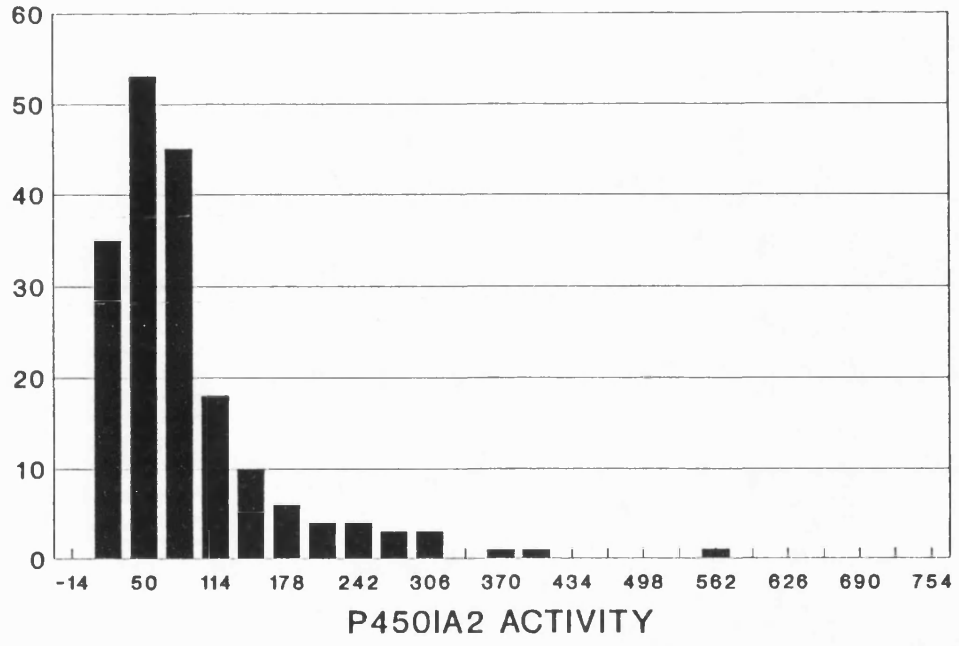


Figure 5.1.12 - Frequency distribution histogram of P450IA2 activity in 184 nonsmoking volunteers.

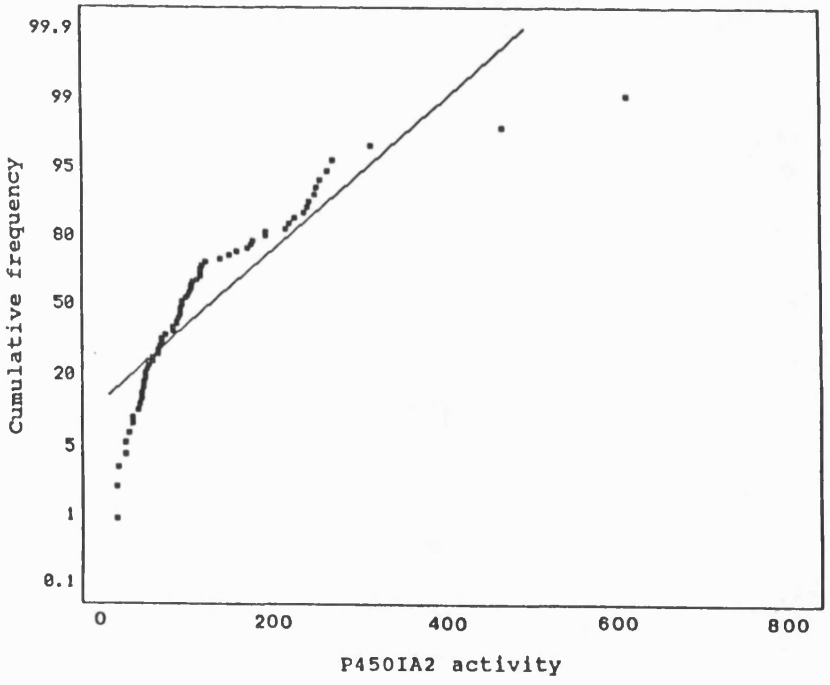


Figure 5.1.13 - Normal probability plot of P450IA2 activity in 70 smoking volunteers.

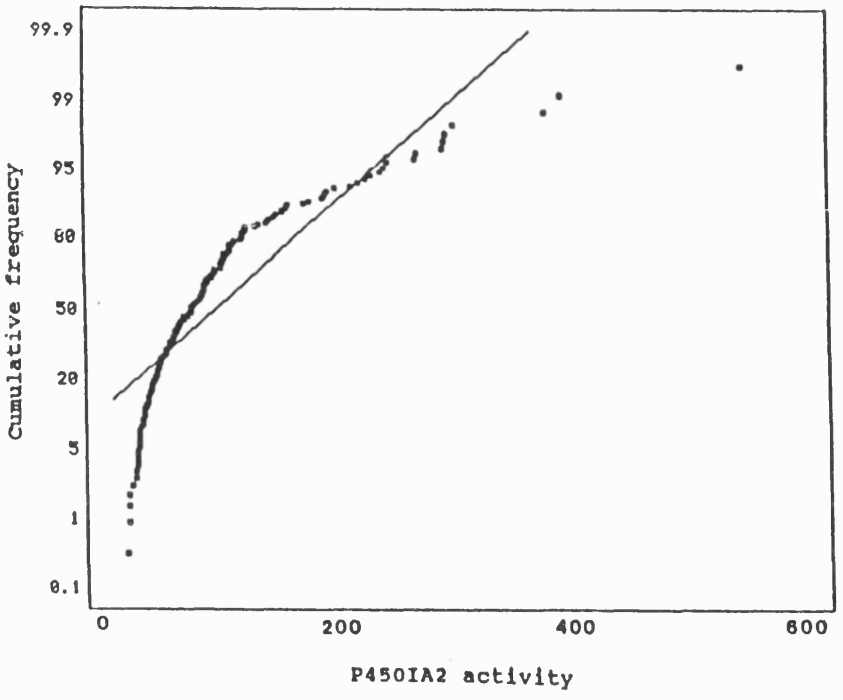


Figure 5.1.14 - Normal probability plot of P450IA2 activity in 184 nonsmoking volunteers.

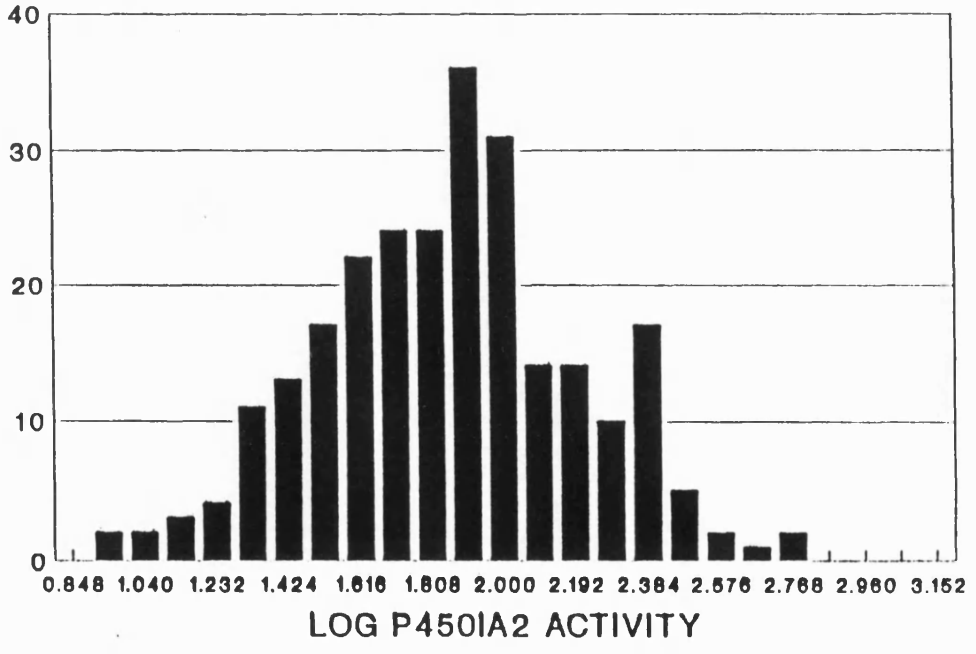


Figure 5.1.15 - Log frequency distribution histogram of P450IA2 activity in 254 volunteers.

Table 5.1.13 - Summary Statistics for P450IA2 activity in smoking and nonsmoking subjects

	Smokers	Nonsmokers
n	70	184
Median	85.48	68.90
Mean	118.66	89.49
Standard error	12.49	5.74
Standard deviation	104.50	77.86
Minimum	7.92	11.58
Maximum	607.62	550.23
Skewness	2.20	2.45
Standardized skewness	7.53	13.56
Kurtosis	6.96	8.53
Standardized kurtosis	11.88	23.13

in this case -unlike P450IA activity, nonsmokers tended to show a less normal distribution than smokers.

The normal probability plot for smokers (Fig.5.1.13) was non-linear (K-S test, $p<0.005$; Chi-square test, $p<0.00005$; Lilly-Fors test, $p<0.001$). Similarly, the normal probability plot for P450IA2 activity in nonsmokers (Fig.5.1.14) was non-linear and possibly bimodal (K-S test, $p<0.00001$; Chi-square test, $p<0.00001$; Lilly-Fors test, $p<0.01$).

The data for P450IA2 activity were also logged in an attempt to detect bimodality of the distributions. The frequency distribution histogram for log P450IA2 activity in 254 control volunteers is shown in Fig.5.1.15. The summary statistics for

this population are shown in Table 5.1.14.

Table 5.1.14 - Summary Statistics for log P450IA2 activity in a population of 254 control volunteers

Skewness	-0.008
Standardized skewness	-0.052
Kurtosis	-0.092
Standardized kurtosis	-0.300

Statistical tests indicate that log P450IA2 activity did not differ significantly from a normal distribution and was unlikely to be bimodally distributed (K-S test, $p>0.5$; Chi-square test, $p>0.1$; Lilly-Fors test, $p>0.05$). The summary statistics for log P450IA2 activity in smokers and nonsmokers are shown in Table 5.1.15.

Table 5.1.15 - Summary Statistics for log P450IA2 activity in smoking and nonsmoking subjects

	Smokers	Nonsmokers
n	70	184
Skewness	-0.42	0.16
Standardized skewness	-1.43	0.89
Kurtosis	0.34	-0.20
Standardized kurtosis	0.58	-0.57

Log P450IA2 activity for smoking subjects was not significantly different from normal (K-S test, $p>0.5$; Chi-square test, $p>0.3$; Lilly-Fors test, $p>0.05$). It also seemed unlikely that log P450IA2 activity in nonsmoking subjects was bimodally distributed (K-S test, $p>0.5$; Chi-square test, $p>0.5$; Lilly-Fors test, $p>0.05$).

As the unlogged data for P450IA2 activity suggested that the distribution was bimodal, a cut-off point was arbitrarily assigned in the frequency distribution to separate the control volunteers in the skewed region with relatively high P450IA2 activity from those with enzyme activities in the normal unskewed part of the distribution. In order to achieve such separation, the apparent antimode at 162 was used (Fig.5.1.9). Any subject with a metabolite ratio greater than 162 was classified as having high P450IA2 activity (Table 5.1.16).

Table 5.1.16 - The proportion of control volunteers with high P450IA2 activity

Group	P450IA2 activity			
	High	%	Low	%
All	42/254	(16.5%)	212/254	(83.5%)
Smokers	19/ 70	(27.1%)	51/ 70	(72.9%)
Nonsmokers	23/184	(12.5%)	161/184	(87.5%)

16.5% of the 254 subjects were found to have high P450IA2 activity. 27.1% of the 70 smokers (Fig.5.1.11) and 12.5% of the

184 nonsmokers (Fig.5.1.12) were found to have high P450IA2 activity. The Chi-square test for comparing proportions indicates that smoking subjects have higher P450IA2 activity than nonsmoking subjects ($p<0.01$).

5.1.9 Discussion

Effect of Environmental Factors

The mean P450IA activity determined in the caffeine test involving collection of 0-8 h urine ($n=127$) was not significantly different from that measured in the test involving collection of a spot urine sample 2-6 h after a CCB ($n=150$), in control volunteers. P450IA2 activity determined in the 0-8 h test was also not significantly different from the activity measured in the 2-6 h test. This was expected following our observation that the caffeine MRs used to determine P450IA and P450IA2 activities were not altered by either the time of urine collection or the or the amount of caffeine consumption (Chapter 4). George *et al* (1986) also found that caffeine pharmacokinetic values were similar whether subjects abstained from methylxanthine- containing products for 1 week prior to the study or if they consumed 6 cups of coffee daily, for 1 week before the study. Values for enzyme activities in the two control groups were thus combined and treated as one group.

During this study, a strong correlation between P450IA and P450IA2 activities was found ($r=0.4228$; $p<0.00001$) which suggests that either P450IA2 catalyses both reactions or if P450IA1 is involved in 1,7-DMX demethylation, it is very closely related to P450IA2. It is known that these isozymes are under close genetic regulation and coordinately expressed. As P450IA1

has been detected in human liver following exposure to certain environmental chemicals (Quattrochi et al, 1985; Wrighton et al, 1986; Jaiswal et al, 1987; Cresteil & Eisen, 1988; Ikeya et al, 1989) and it is possible for P450IA1 to metabolise 1,7-DMX (as discussed in Chapter 3), it may be the case that P450IA1 contributes to a proportion of 1,7-DMX demethylation.

No correlation between subject age and either the degree of P450IA or P450IA2 activity existed. Grant et al (1983b) also found that caffeine metabolite recoveries and profiles in healthy volunteers (aged 16 to 65 y) was unrelated to subject age. It is known that caffeine biotransformation is altered in the young (Ginchansky & Weinberger, 1977; Aldridge et al, 1979: Grygiel & Birkett, 1980; Tserng et al, 1981; Pons et al, 1988) and elderly (Schnegg & Lauterburg (1986) but it was unlikely that caffeine metabolism would be affected in the present study as only 22 of the 277 healthy subjects studied were 65 y of age or older.

Decreased hepatic clearance of drugs in old age is related to loss of liver volume rather than changes in the function of the microsomal enzymes and should not therefore be detected by metabolite ratios. Indeed there is no difference in drug metabolism in vitro by microsomes isolated from young and aged livers (Varagnolo et al, 1989). Hence, measurements of caffeine clearance may be of more use than metabolite ratios when trying to quantitate liver function in the elderly (Renner et al, 1984; Varagnolo et al, 1989).

BMI had no effect on P450IA or P450IA2 activities, in agreement with a study by Grant et al (1983a). Grant et al (1983b) also

found no correlation between subject weight and caffeine metabolite ratios. It is possible that obesity might alter caffeine disposition, as Kamimori et al (1987) showed that obese volunteers had a longer caffeine half life than lean volunteers. Only three volunteers however, were studied for each group. As only a few of the volunteers in our study had BMI values outside the ideal weight range, the effect of obesity could not be examined. Caraco et al (1991) also found that caffeine pharmacokinetic parameters were not altered in severely obese subjects.

P450IA and IA2 activities were not found to be altered by subject gender. This was the case whether males were compared to the whole female population including OCS and non-OCS users, or to the non-OCS user females only. The observation that subject gender has no effect on caffeine metabolism is in agreement with Patwardhan et al (1980) who found that pharmacokinetic parameters were similar between males and females. Callahan et al (1983) and Grant et al (1983b) also found that caffeine metabolism was not significantly different between males and ovulating females.

When the effect of cigarette smoke was investigated, it was found that it increased P450IA and P450IA2 activities to a large extent. However, a slight difference in the extent of induction was observed between the sexes, as following induction by cigarette smoke, the females tended to have higher enzyme activities than the males. In fact, while female smokers had significantly higher P450IA and P450IA2 activities than female nonsmokers, this was not the case for males. Smith et al (1990) also found a sex difference in the susceptibility to

polyhalogenated biphenyl (PHB) induced hepatic uroporphyria in rats. Whereas the total P450 content was higher in control or induced male rats compared to females, hepatic P450IA1 and IA2 were more highly induced in female rats. As female rats are considerably more sensitive to induction of porphyria, their results were consistent with the association of P450IA with porphyria development. In the present study, the increased responsiveness of females to the inductive effect of cigarette smoke was likely to be due to the effect of subject gender and not age or BMI, as no significant difference in either age or BMI existed between the sexes. Overall however, no significant difference in mean enzyme activities between males and females existed, whether smoking, nonsmoking or whole populations were compared.

The fact that smokers had significantly higher enzyme activities than nonsmokers, despite the large interindividual differences in caffeine metabolism that exist, indicates that smoking has a large effect on the enzymes of interest. A similar result was found by Kotake et al (1982) who found that the rate of caffeine metabolism in the caffeine breath test (CBT) was doubled in smokers compared to nonsmokers. Several other studies also showed that cigarette smoke markedly affected caffeine metabolism (Parsons & Neims, 1978; May et al, 1982; Campbell et al, 1987; Sesardic et al, 1990) and had a similar effect on theophylline disposition (Jenne et al, 1975; Hunt et al, 1976; Grygiel et al, 1984; Cusack et al, 1985). A study by Brown et al (1988) investigated the effects of tobacco abstinence on the pattern of caffeine metabolism in 9 habitual smokers. Caffeine metabolism was considerably higher while the volunteers were

smoking, whereas a 60% decrease in caffeine clearance occurred after only 3-4 days of cigarette abstinence. Smoking was found to accelerate both primary and secondary demethylation pathways, in agreement with our results, while the ratio of 1,7-DMU/ 1,7-DMX did not differ between smoking and nonsmoking phases.

The increased P450IA2 activity that we observed in smokers compared to nonsmokers is in agreement with previous studies (Pantuck et al, 1974; Sesardic et al, 1988) which showed that phenacetin O-deethylation, catalysed by P450IA2 activity in human liver, was induced by cigarette smoke. Earlier work on AHH activity (a proportion of which is catalysed by P450IA1), found that smokers had higher AHH activity than nonsmokers in extrahepatic tissues, including placenta (Nebert et al, 1969; Vaught et al, 1979; Pasanen & Pelkonen, 1981), human monocytes (Jett et al, 1978; Rudiger et al, 1980) and lymphocytes (Karki & Huhti, 1978; Jett et al, 1978; Hopkin & Evans, 1980; Gurtoo et al, 1983). EROD activity of placenta (Pelkonen et al, 1979) and 7-ethoxycoumarin activity of human lung samples (Oesch et al, 1980), proportions of which are catalysed by P450IA1, were also induced by cigarette smoke, while placental P450IA1 activity was induced in Chinese women exposed to contaminated rice oils containing PCBs (Wong et al, 1986). P450IA1 can also be induced in human breast cell lines following exposure to airborne particles (Roepstorff et al, 1990). Interestingly, AHH activity in human liver is not increased by cigarette smoking (Vahakangas et al, 1983; Pelkonen et al, 1979); probably because it is catalysed by P450IIC and P450IIIA (Kawano et al, 1987) and not by P450IA1 (Fujino et al, 1984), as occurs in human placenta (Fujino et al, 1982; Wong et al, 1986; Pasanen et al, 1989). As

caffeine is metabolised mainly in the liver and it is uncertain whether P450IA1 is involved in its metabolism, it was not possible to assess P450IA1 levels extrahepatically. However, demethylation of 1,7-DMX in the liver (a proportion of which may involve P450IA1) was also increased in smokers compared to nonsmokers in the present study.

OCS or HRT administration was found to have only a small effect on caffeine metabolism because although P450IA and P450IA2 activities tended to be lower in OCS-user females than women not using OCS, the results were not significantly different. Other studies found a significantly lower plasma clearance of caffeine and a significantly prolonged caffeine half-life in OCS-user females (Patwardhan et al, 1980; Abernethy & Todd, 1985; Meyer et al, 1988). A similar result was observed for theophylline disposition (Gardner et al, 1983). Also, Campbell et al (1987) found that nonsmoking OCS users had lower mean caffeine metabolite ratios than nonusers. As caffeine metabolism is prolonged during pregnancy (Knutti et al, 1981) it is possible that steroid administration suppresses the MFO system involved in caffeine metabolism (Patwardhan et al, 1980).

Females using OCS/HRT were studied further for their smoking status to see whether this altered the results. In nonsmoking females, P450IA activity was not significantly different in women who were OCS/HRT or non-OCS users. This was also the case for P450IA2 activity. However, in smoking females, P450IA activity was significantly lower in OCS/HRT users than in non-OCS users. In contrast, this relationship was not found for P450IA2 activity.

Although OCS or HRT administration per se did not appear to

alter caffeine metabolism, it did have an effect on P450IA activity, particularly in the smoking population, as concurrent OCS/HRT use actually inhibited the effect of cigarette smoke on enzyme activities. That is, the smokers had a similar P450IA activity to nonsmokers if women were taking these steroids, whereas in non-OCS females, smokers had a much greater P450IA activity than nonsmokers. A similar finding was observed by Gardner et al (1983), who studied the combined effects of cigarette smoke and OCS administration on caffeine metabolism.

The modest effect of OCS on caffeine metabolism observed in the present study may be explained by the wide interindividual range in enzyme activities observed. Variation due to genetic factors, may well override the inhibitory effects of OCS administration and results would suggest that OCS administration does not affect P450IA or P450IA2 to as great an extent as other environmental factors, such as cigarette smoking.

Effect of Genetic host factors

Large interindividual differences in caffeine metabolism exist in a group of control volunteers. Frequency distributions for both P450IA and IA2 were positively skewed and statistical tests performed on the data indicated that P450IA and P450IA2 activities strongly deviated from a normal distribution. Even when the smoking subjects were studied separately, in order to try and reduce some of the variability observed, P450IA activity still ranged 103-fold for smokers and 19-fold for nonsmokers. P450IA2 activity ranged 77 -fold for smokers and 48-fold for nonsmokers.

The possibility of genetic differences in AHH inducibility in

man was first described by Kellermann et al (1973a) who claimed that humans could be separated into three groups of low (45%), intermediate (46%) and high (9%) inducibility. Gahmberg et al (1979) also found that 15% of a Finnish population exhibited high AHH inducibility, although they obtained a bimodal distribution for AHH activity. It was also found by other workers that considerable variation in AHH activity existed in human tissues, including lymphocytes (Arnott et al, 1979; Gurtoo et al, 1983), human pulmonary alveolar macrophages (Cantrell et al, 1973), placenta (Pelkonen et al, 1979), bronchi (Harris et al, 1976), lung biopsy tissue (Oesch et al, 1980; Roberts et al, 1986), bone marrow cells (Brown et al, 1976) and human foreskin (Levin et al, 1972). Human lung cytosol samples were also found to exhibit striking heterogeneity in Ah receptor concentrations (Roberts et al, 1986). Jaiswal et al (1985) showed genetic differences in the inducibility of P450IA1 mRNA in human lymphocytes and polymorphisms of the P450IA1 gene were then discovered (Jaiswal & Nebert, 1986). Three P450IA1 genotypes existed in 104 healthy subjects - the frequency of which showed excellent agreement with the trimodal AHH inducibilities (Kawajiri et al, 1990).

In order to detect whether bimodality in frequency distributions of data existed in the present studies, the activities were logged and studied further in the form of histograms and cumulative distributions. Log transformation of data often allows clearer visualisation of different phenotypes and therefore clearer bimodality of distributions under monogenic control (Jackson et al, 1986). The frequency distributions for

P450IA activity was still skewed when the logged data were studied, and the Lilly-Fors test which tests specifically for bimodality of distributions, showed that P450IA activity was likely to be bimodal. As no clear subgroups of individuals with high P450IA activity were evident, a cut-off point in the frequency distribution at a ratio of 26 was arbitrarily assigned, to separate those subjects in the skewed region of the distribution with high P450IA activity. In this way 9% of control volunteers in the present study had high P450IA activity, in good agreement with results from groups studying AHH activity (Kellermann et al, 1973a; Kawajiri et al, 1990).

Conversely, P450IA2 activity appeared to be normally distributed when the data were logged, which again suggests differences in the two pathways of caffeine metabolism. Large differences in P450IA2 activity have been described (Wrighton et al; 1986; Davies et al; 1987). The metabolism of aminobiphenyl (Butler et al, 1989a) and 2-naphthylamine (Hammons et al, 1985) which is catalysed by P450IA2, varied 30-60 fold and the metabolism of phenacetin varied 180-fold in only 28 human liver samples (Shimada et al, 1989; Sesardic et al, 1988). In addition, caffeine 3-demethylation, which is catalysed by P450IA2 in human liver was found to vary greatly (Butler et al, 1989a; Sesardic et al, 1990). Most of these workers have suggested the possibility of a genetic polymorphism in P450IA2 to explain such large interindividual variation.

In accordance with other studies, the population was divided into arbitrary groups with high and low P450IA2 activity, using the metabolite ratio of 162 as the cut-off point. In this way, 16.5% of healthy subjects were found to have high P450IA2

activity in this study. Bartsch et al (1990) found that 25% of 100 healthy white males had high P450IA2 activity, using the paraxanthine/ caffeine metabolite ratio.

Although modes or subgroups of individuals with different P450IA and P450IA2 activities are not clearly evident, the possibility of a pharmacogenetic polymorphism in the activity of one or both of these enzymes cannot be ruled out. For many genetic traits there is considerable variation within phenotypes either for genetic reasons or because of environmental factors (Jackson et al, 1989a). Such variability may obscure the separation between phenotypes thereby complicating the detection of a polymorphism. If it were possible routinely to genotype individuals, these problems would be overcome. However, the molecular biology of the enzymes involved suggests that the underlying genetic basis for their activity is complex (Guengerich et al, 1987). Therefore, polymorphisms are still being detected on the basis of phenotypic expression of enzyme activity, even though statistical proof of bimodality in population distributions is difficult.

Although genetic polymorphism causes marked variability in the response to drugs and environmental chemicals (Ayesh & Smith, 1989), environmental factors are particularly important for the variability of drug dispostion involving the cytochromes P450, as their activity is greatly altered by environmental exposure to inducing and inhibiting agents. As distributions for P450IA and P450IA2 activities do not show clear subgroups of individuals with high and low activity, it is difficult to distinguish between the environmental and genetic factors

contributing to the observed variability. Nevertheless, in order to try and determine the degree of variation in P450IA and IA2 activities due to genetic factors, smoking and nonsmoking subjects were studied separately in the present study, as cigarette smoke is known to be one of the largest environmental contributions to P450IA and IA2 variability.

The 103-fold variation in P450IA activity in subjects who were all smokers indicates that the degree of inducibility of this enzyme is very different, even in subjects who may smoke the same number of cigarettes - and therefore be exposed to PAH to a similar degree. Several other workers also found that AHH activity varied markedly between individuals who smoked a similar number of cigarettes (Welch et al, 1969; Harris et al, 1976; Vaught et al, 1979; Sabadie et al, 1981). Some of this variation may be attributed to different amounts of tobacco smoked or to varying patterns of inhalation or airflow in the bronchial tree, which would clearly alter the dose of carcinogen delivered to the bronchial mucosa and peripheral lymphocytes (Hopkin & Evans, 1980). However, although plasma thiocyanate is higher in smokers than nonsmokers (Vahakangas et al, 1983), the levels correlated weakly with placental AHH activity of smoking women, which suggests that there is likely to be a genetic component controlling AHH variability (Pelkonen et al, 1979). Statistical tests performed on the logged data, suggest that P450IA activity is bimodal for smoking subjects.

The nonsmokers only exhibited a 19-fold range in P450IA activity, which suggests that it is the degree of inducibility that varies more markedly between subjects than basal enzyme levels. Kellermann et al (1973b) found that basal AHH levels

varied within a smaller range than induced levels, although basal and induced AHH levels were highly correlated. Nebert (1991) also suggested that differences in P450IA1 and P450IA2 activities are likely to represent differences in the the human Ah receptor gene controlling inducibility of the enzymes.

In contrast to smokers, bimodality in log P450IA activity for nonsmokers was not evident. The distributions for logged P450IA2 activity were unimodal whether smokers, nonsmokers or the whole population were studied.

Nevertheless, considerable evidence in favour of a large genetic effect leading to variation in enzyme activities exists and results from the reproducibility tests, discussed in Section 4.1, further support this theory. P450IA and P450IA2 activities were highly reproducible when the same caffeine test was performed three months apart, whether subjects were smokers or nonsmokers. Such a large genetic effect may explain why some of the subjects with high enzyme activities were in fact nonsmokers. It may be possible then, to use the caffeine test to measure P450IA and P450IA2 activities, in order to study the degree of inducibility that occurs in smokers and to determine the nonsmoking subjects who have particularly high enzyme activities.

5.2 P450IA AND P450IA2 ACTIVITIES IN PATIENTS WITH CARCINOMA OF THE LUNG, HEAD AND NECK

5.2.1 Introduction

Given that large variations in P450IA and IA2 enzyme activities exist in healthy subjects and that these enzymes can be induced by cigarette smoking, P450IA and IA2 activities were measured in patients with carcinoma of the lung, head and neck. Lung cancer patients have been shown to exhibit the high P450IA1 inducibility phenotype (Kawajiri et al, 1990), which may be the biochemical basis for the observation that lung cancer occurs more frequently in both smoking and nonsmoking relatives of lung cancer patients (Anderson, 1975). Thus the differential susceptibility of the human population to chemical carcinogens may be partially attributed to individual differences in carcinogen metabolism. Examination of the levels of such enzymes in humans may contribute to an understanding of the genetic and environmental factors which affect the susceptibility of an individual for cancer.

By giving caffeine as a probe drug to a group of patients with carcinoma of the lung, head and neck, and measuring the metabolites of interest in urine, P450IA and P450IA2 activities were determined and compared to those from a population of control volunteers (Section 2.3.2).

5.2.2 Results

The mean enzyme activities for the patients with cancer are shown in Table 5.2.1. When compared to control subjects, the patients with lung cancer had a highly significantly greater P450IA activity ($p<0.01$) (Fig.5.2.1). However, there was no

Table 5.2.1 - P450IA and P450IA2 activities in patients with carcinoma of the lung, head and neck and healthy subjects

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Control	277	11.64	14.52 ± 0.87	1.15 -130.33
	Cancer	51	15.32	18.16 ± 1.71	5.13 - 62.69
P450IA2	Control	254	74.68	97.29 ± 5.45	7.92 -607.62
	Cancer	51	65.91	92.30 ±12.55	17.39 -411.97

significant difference in P450IA2 activity between the patients with lung cancer and the control subjects ($p>0.5$) (Fig.5.2.2). The mean age of 51 patients with carcinoma of the lung, head and neck was 67.92 ± 1.41 y. This was significantly higher than the mean age of 40.24 ± 0.95 y for 277 control volunteers ($p<0.0001$).

5.2.3 Effect of Cigarette Smoke

P450IA and P450IA2 activities were studied in 51 patients with carcinoma of the lung, head and neck of known smoking status; 16 were smokers and 35 were nonsmokers. The results are shown in Table 5.2.2. Athough smokers did tend to have a higher P450IA activity than nonsmokers, results were not significantly different in the patients with lung cancer whether they were smokers or nonsmokers ($p>0.3$).

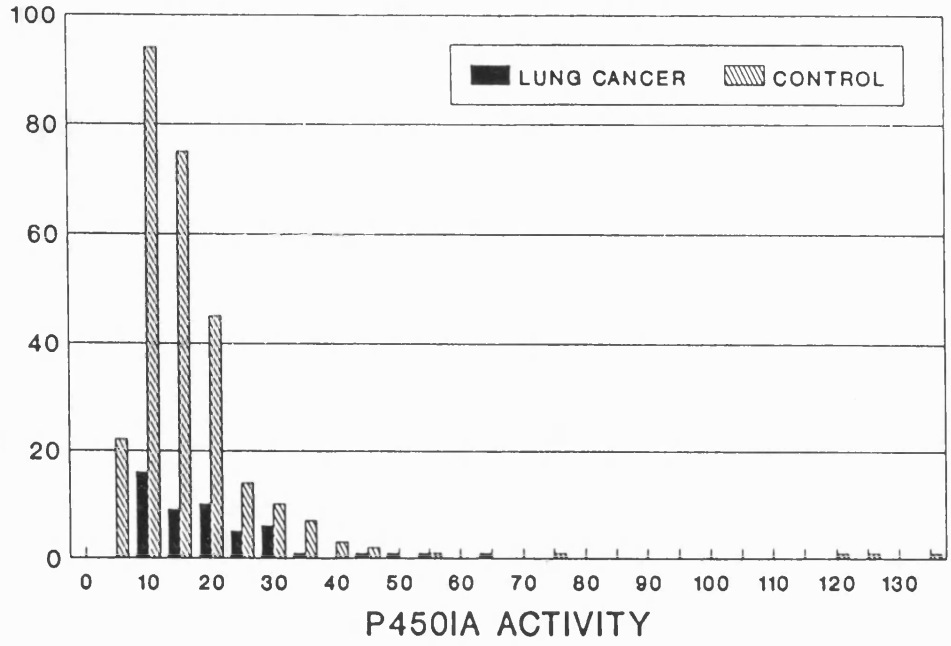


Figure 5.2.1 - P450IA activity in patients with carcinoma of the lung, head and neck, compared to controls.

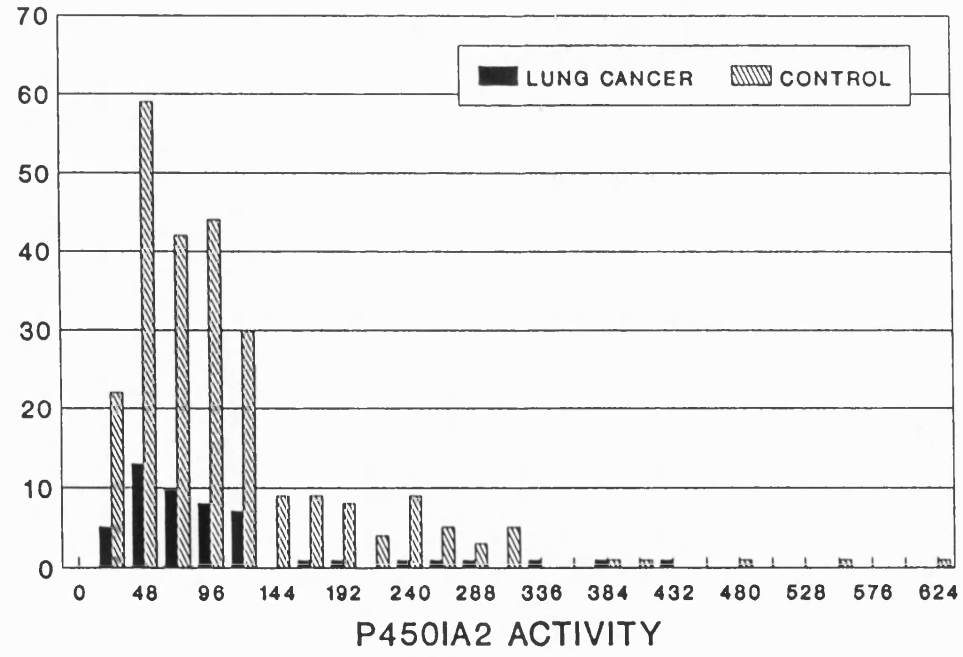


Figure 5.2.2 - P450IA2 activity in patients with carcinoma of the lung, head and neck, compared to controls.

Table 5.2.2 - P450IA and P450IA2 activities in smoking and nonsmoking cancer patients

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Smokers	16	17.23	20.00 ± 3.12	6.96 - 49.51
	Nonsmokers	35	14.89	17.33 ± 2.06	5.13 - 62.69
P450IA2	Smokers	16	102.03	133.32 ±24.47	42.99 -371.78
	Nonsmokers	35	51.57	73.56 ±13.56	17.39 -411.97

In contrast, patients with lung cancer who were smokers had a higher P450IA2 activity than nonsmokers ($p<0.005$). Enzyme activities between smokers and nonsmokers from each population were compared separately. The results are shown in Table 5.2.3. The smoking cancer patients had a higher mean P450IA activity than smoking control volunteers, but not significantly so ($p>0.05$). Nonsmoking patients with lung cancer had significantly higher P450IA activity than nonsmoking controls ($p<0.005$). In fact, P450IA activity in nonsmoking cancer patients tended to be higher than that of control smokers.

P450IA2 activity in smoking cancer patients was not significantly different to that of control smokers ($p>0.1$). P450IA2 activity in nonsmoking cancer patients was also not significantly different from that of nonsmoking controls ($p>0.1$).

Table 5.2.3 - P450IA and P450IA2 activities in smoking and nonsmoking cancer patients and healthy subjects

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Smokers				
	-control	70	13.76	16.55 ±1.94	1.15 - 118.46
	-cancer	16	17.23	20.00 ±3.12	6.96 - 49.51
	Nonsmokers				
	-control	185	10.09	12.21 ±0.57	2.95 - 54.89
	-cancer	35	14.89	17.33 ±2.06	5.13 - 62.69
P450IA2	Smokers				
	-control	70	85.48	118.66 ±12.49	7.92 - 607.62
	-cancer	16	102.03	133.32 ±24.47	42.99 - 371.78
	Nonsmokers				
	-control	184	68.90	89.49 ±5.74	11.58 - 550.23
	-cancer	35	51.57	73.56 ±13.56	17.39 - 411.97

5.2.4 Effect of Cancer Cell Type

P450IA and P450IA2 activities were studied in 43 patients with carcinoma of the lung, head and neck of known cell type. 30 patients had tumors of squamous cell origin; 10 were of oat cell origin; 1 tumor was of swan cell type and 2 were adenocarcinomas.

The enzyme activities for each group are shown in Table 5.2.4. The patient who had a tumor of the swan cell type had the lowest P450IA and P450IA2 activities measured. Patients with the three

Table 5.2.4 - P450IA and P450IA2 activities in patients with carcinoma of the lung, head and neck of different cell type

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Squamous	30	16.76	18.31 ± 2.29	5.61 - 62.69
	Oat	10	23.27	23.17 ± 3.00	12.47 - 42.60
	Adenocarcinoma	2	16.18	16.18 ± 1.29	14.89 - 17.47
	Swan	1	7.14	7.14	
P450IA2	Squamous	30	55.10	96.35 ±18.26	22.51 -411.97
	Oat	10	88.25	123.66 ±27.64	51.57 -318.59
	Adenocarcinoma	2	75.96	75.96 ± 2.92	73.04 - 78.87
	Swan	1	17.39	17.39	

other types of cell tumors seemed to have comparable enzyme activities. As only two patients presented with adenocarcinoma, only results for the squamous and oat cell types were compared statistically. Although patients with lung, head and neck tumors of oat cell origin tended to have higher P450IA and P450IA2 activities than those with tumors of squamous cell types, there was no significant difference between the two groups ($p>0.1$) for either P450IA activity or P450IA2 activity.

5.2.5 Effect of Tumor Location

P450IA and P450IA2 activities were studied in 41 patients with carcinoma of the bronchus and in 10 patients with cancer of the

head and neck. The enzyme activities for each group are shown in Table 5.2.5. P450IA activity was virtually identical in patients with cancer of the bronchus and cancer of the head and neck and the results were not significantly different ($p>0.5$).

Table 5.2.5 - P450IA and P450IA2 activities in patients with carcinoma of the bronchus and cancer of the head and neck

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Bronchus	41	15.32	18.03 ± 1.92	5.13 - 62.69
	Head & neck	10	15.71	18.70 ± 3.96	8.34 - 50.91
P450IA2	Bronchus	41	69.40	98.64 ±14.48	17.39 -411.97
	Head & neck	10	36.25	66.36 ±23.43	22.51 -266.30

Bronchial cancer patients alone had a significantly higher P450IA activity than the control population ($p<0.05$). Even the small group of patients with head and neck cancer ($n=10$) had a higher P450IA activity than control subjects ($p=0.01$).

P450IA2 activity tended to be higher in patients with bronchial carcinoma than head and neck patients but results between the two groups were not significantly different ($p>0.1$). Therefore, P450IA2 activity was not significantly different between controls and cancer patients, whether bronchial or head and neck cancer.

5.2.6 Genetic Control of P450IA and P450IA2 Activities

P450IA Activity

The frequency distribution histogram for P450IA activity in 51 patients diagnosed with carcinoma of the lung, head and neck is depicted in Fig.5.2.3. The summary statistics for this distribution are shown in Table 5.2.6.

Table 5.2.6 - Summary Statistics for P450IA activity in 51 patients with carcinoma of the lung, head and neck

Median	15.32
Mean	18.16
Standard error	1.71
Standard deviation	12.23
Minimum	5.13
Maximum	62.69
Skewness	1.76
Standardized skewness	5.12
Kurtosis	3.57
Standardized kurtosis	5.22

12 fold variation in P450IA activity exists in 51 patients with lung cancer. It can be seen from the histogram and Table 5.2.6 that the distribution is strongly positively skewed and shows a degree of kurtosis. The standardized coefficients are both ouside the range of -2 to +2 that would be observed for a normal distribution and therefore indicate that the data may deviate significantly from normality. This is confirmed by the Chi-

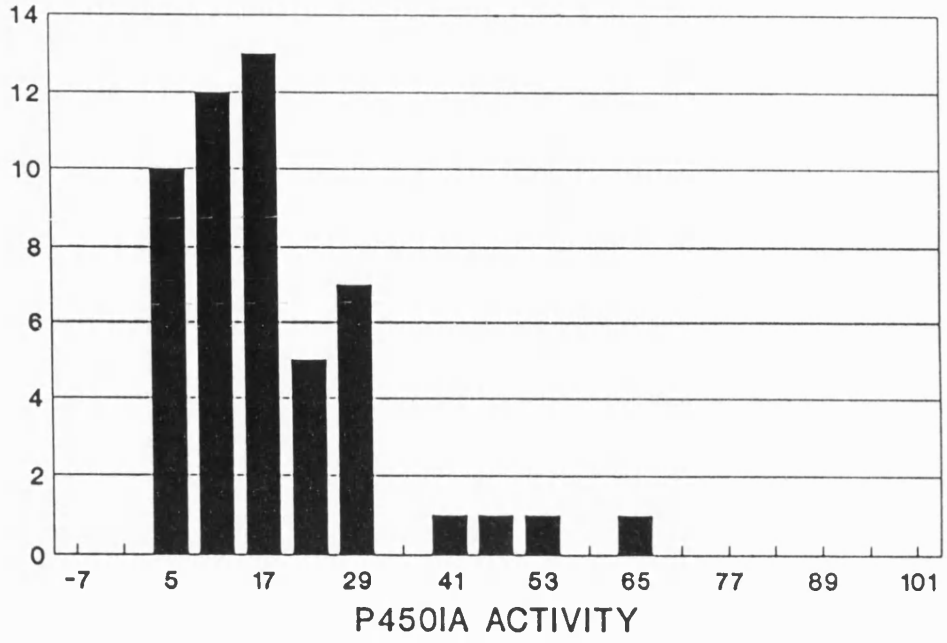


Figure 5.2.3- Frequency distribution histogram of P450IA activity in patients with carcinoma of the lung, head and neck

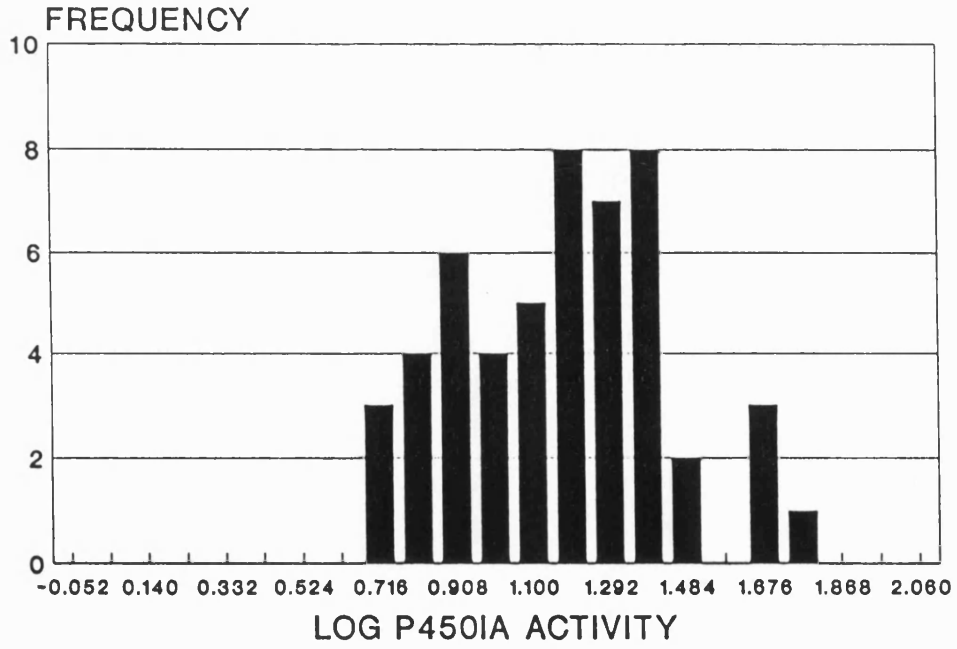


Figure 5.2.4 - Log frequency distribution histogram of P450IA activity in 51 patients with carcinoma of the lung, head and neck.

-square and the Lilly-Fors test which indicate that P450IA activity was highly significantly different from that expected from a normal distribution.

As the data show a strong degree of skewness and kurtosis, the data were logged and re-examined in order to detect bimodality of the distribution. The frequency distribution histogram for log P450IA activity in 51 patients with carcinoma of the lung, head and neck is shown in Fig.5.2.4. The distribution is slightly positively skewed but statistical tests performed on the data also suggested that P450IA activity was unimodal.

21.6% of the patients with cancer of the lung, head and neck had high P450IA activity (Fig.5.2.3). This was significantly higher than the proportion (9%) of control volunteers with high P450IA activity ($p<0.01$). 31.3% of the smoking cancer patients had high P450IA activity, compared to 11.4% of the control smokers ($p<0.05$) and 17.1% of the nonsmoking cancer patients had high P450IA activity, compared to only 6.5% of the control nonsmokers ($p<0.05$). The proportion of smoking and nonsmoking cancer patients with high P450IA activity was not significantly different ($p>0.05$).

P450IA2 Activity

The frequency distribution histogram for P450IA2 activity in 51 patients diagnosed with carcinoma of the lung, head and neck is depicted in Fig.5.2.5. The summary statistics for this distribution are shown in Table 5.2.8.

Table 5.2.7 - The proportion of lung, head and neck cancer patients with high P450IA activity

Group	P450IA activity			
	High	%	Low	%
Cancer	11/51	(21.6%)	40/51	(78.4%)
-Smokers	5/16	(31.3%)	11/16	(68.7%)
-Nonsmokers	6/35	(17.1%)	28/35	(82.9%)
Control	25/277	(9.0%)	252/277	(91.0%)
-Smokers	8/ 70	(11.4%)	62/ 70	(88.6%)
-Nonsmokers	12/185	(6.5%)	173/185	(93.5%)

Table 5.2.8 - Summary Statistics for P450IA2 activity in 51 patients with carcinoma of the lung, head and neck

Median	65.91
Mean	92.30
Standard error	12.55
Standard deviation	89.65
Minimum	17.39
Maximum	411.97
Skewness	2.14
Standardized skewness	6.23
Kurtosis	4.29
Standardized kurtosis	6.25

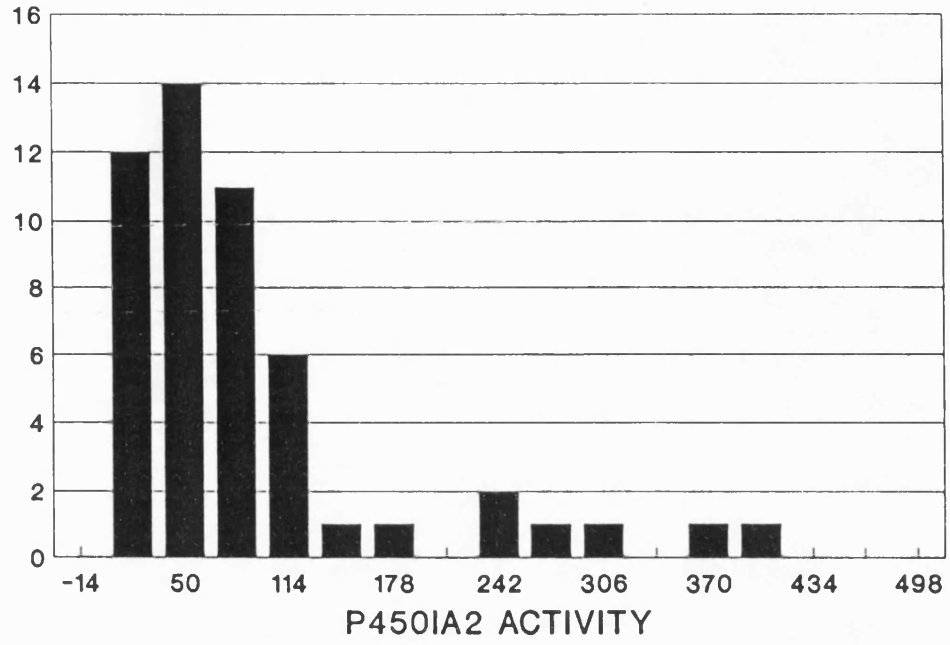


Figure 5.2.5 - Frequency distribution histogram of P450IA2 activity in 51 patients with carcinoma of the lung, head and neck.

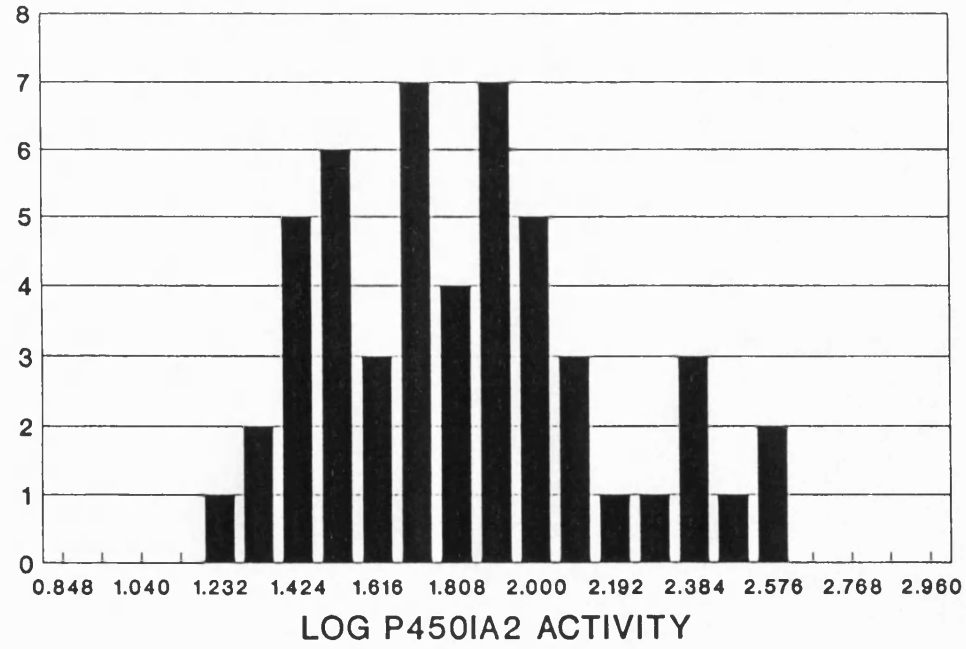


Figure 5.2.6 - Log frequency distribution histogram of P450IA2 activity in 51 patients with carcinoma of the lung, head and neck.

24-fold variation in P450IA2 activity existed. It can be seen from the histogram and Table 5.2.7 that the distribution is strongly positively skewed and shows a degree of kurtosis. The standardized coefficients suggest that the data may deviate significantly from normality. This is confirmed by both the Chi-square and K-S test results, which are significantly different from normal. In addition the Lilly-Fors test suggested that P450IA2 activity may form a bimodal distribution.

The frequency histogram for log P450IA2 activity in 51 patients with carcinoma of the lung, head and neck is depicted in Fig.5.2.6.

The distribution for log P450IA2 activity is positively skewed to a greater extent than that for P450IA activity, but the statistical tests performed on the data suggest that P450IA2 activity is unimodal, even when the smokers and nonsmokers were considered separately.

The proportion of cancer patients with high P450IA2 activity (ratio >162) is shown in Table 5.2.9.

13.7% of the patients with cancer of the lung, head and neck were found to have high P450IA2 activity (Fig.5.2.5). This was not significantly different from the proportion (16.5%) of control subjects with high P450IA2 activity ($p>0.05$). The proportion of smoking (25%) and nonsmoking cancer patients (8.6%) with high P450IA2 activities were not significantly different from those found in the control smoking (27.1%) and nonsmoking (12.5%) subjects ($p>0.05$).

Table 5.2.9 - The proportion of lung, head and neck cancer patients with high P450IA2 activity

Group	P450IA2 activity			
	High	%	Low	%
Cancer	7/51	(13.7%)	44/51	(86.3%)
-Smokers	4/16	(25.0%)	12/16	(75.0%)
-Nonsmokers	3/35	(8.6%)	32/35	(91.4%)
Control	42/254	(16.5%)	212/254	(83.5%)
-Smokers	19/ 70	(27.1%)	51/ 70	(72.9%)
-Nonsmokers	23/184	(12.5%)	161/184	(87.5%)

5.2.7 Discussion

Early studies on the relationship between enzyme activities and cancer susceptibility showed that the AHH enzyme could metabolise BP and other such PAH, resulting in the formation of DNA adducts (Digiovanni et al, 1983) and cancer initiation in animal (Nebert & Felton, 1975; Heidelberger et al, 1980) and human tissues (Yang et al, 1977). A good correlation existed between BP activation, the degree of DNA binding and sensitivity to the carcinogen in vitro (Grunberger & Theall, 1983). The extent of the carcinogenicity of PAH and aromatic amine compounds appeared to correlate with the degree of induction of the P450IA enzymes (Creaven & Parke, 1966; Ayrton et al, 1990a,b). Following the observation that large variations in AHH activity existed in human lymphocytes (Kellermann et al, 1973a) such that a trimodal distribution was formed, several workers

measured AHH activity in patients with lung cancer. Kellermann et al (1973b) found that 30% of bronchial carcinoma patients were of the high AHH inducibility phenotype compared to 9% in healthy subjects. Several other groups also found that AHH activity was higher in human lymphocytes from patients with lung cancer than healthy controls (Coombes et al, 1976; Guirgis et al, 1976; Karki & Huhti, 1978; Emery et al, 1978; Gahmberg et al, 1979; McLemore et al, 1979; Arnott et al, 1979; Kouri et al, 1982) and that the number of BP-DNA adducts formed in these patients was higher, as a result of their increased AHH activity (Rudiger et al, 1980).

In addition, studies on P450IA1 itself (Kawajiri et al, 1990) have shown that individuals with the homozygous rare allele of the polymorphism for the CYPIA1 gene were at a 3-fold risk for lung cancer and at a 5- fold risk for the squamous cell type in particular. Moreover, the proportion of patients with the rare allele was the same as that with the high AHH inducibility phenotype (30%) studied by Kellermann (1973b). Nakachi et al (1991) found that patients with the homozygous rare P450IA1 allele contracted lung cancer after smoking fewer cigarettes than other genotypes possibly due to differences in metabolic activation of carcinogens between different genotypes. More evidence in favour of this comes from a recent study by Kawajiri et al (1991) who found that the polymorphism in CYPIA1 cosegregated with the inducibility phenotype of P450IA1, which suggests that the susceptible genotype may have increased metabolism of procarcinogens in cigarette smoke. Therefore, the risk of lung cancer in individuals with the rare CYPIA1 allele may be explained by their high P450IA activity. The importance

of P450IA in the cancer process is shown by the fact that guinea pigs and steppe lemmings which are resistant to chemical carcinogens, are also resistant to P450IA induction by PAH (Ioannides & Parke, 1987).

P450IA activity in the present study was found to be considerably higher in patients with lung cancer than controls, even in the presence of 113-fold variation in P450IA activity in healthy subjects. It is tempting to speculate that the P450IA isozyme involved in 1,7-DMX N-demethylation is in some way related to the AHH enzyme, and may thus be used as a marker of susceptibility for cancer of the lung, head and neck.

P450IA activity was not found to alter between smoking and nonsmoking patients with carcinoma of the lung, head and neck. P450IA activity in smoking cancer patients was not significantly different from that in healthy smokers but the proportion of smoking patients with high P450IA activity (31.3%) was significantly higher than that in healthy smokers (11.4%). In contrast, the nonsmoking cancer patients had a much higher P450IA activity than nonsmoking healthy subjects and the proportion of these patients with high P450IA activity (17.1%) was much higher than that in healthy nonsmokers (6.5%). These findings strongly suggest that factors other than cigarette smoke are contributing to the high P450IA activity observed in the cancer subjects. Most of the patients classified as nonsmokers in this study were in fact exsmokers for at least one month prior to the time of the caffeine test. As Cantrell et al (1973) showed that AHH activity returned to basal levels within 2 months cessation of smoking and Brown et al (1988) found that caffeine metabolite patterns returned to basal levels following

3-4 days of cigarette abstention, high P450IA activities in the nonsmoking cancer population are unlikely to be due to the effects of cigarette smoke.

When the frequency distribution for P450IA activity was studied, the cut-off ratio of 26 determined from 277 healthy volunteers fitted well with the distribution for the cancer patients. 21.6% of the cancer population had high P450IA activity compared to only 9% of control subjects. This is slightly less than the proportion of lung cancer patients with high AHH inducibility (Kellermann et al, 1973b) and the rare CYPIA1 allele (Kawajiri et al, 1990). Nonetheless, patients with cancer of the lung, head and neck -particularly the nonsmokers had a much greater P450IA activity than the control population, in agreement with Gahmberg et al, (1979).

The 12-fold range in P450IA activity for the patients with cancer is similar to that found for AHH in lung tissue by Sabadie et al (1981) and supports the idea that the patients with cancer are a more homogeneous group, with high P450IA activity, then control subjects.

The possibility that high P450IA activity is due to the presence of the tumour itself cannot be ruled out but seems unlikely as Idle et al (1981) found that the metabolism of mephenytoin and antipyrine were unaltered in cancer cases, compared to controls.

P450IA2 activity was not found to alter significantly between cancer patients and controls. The proportion of patients with high P450IA2 activity (13.7%) was less than that in control subjects (16.5%). In fact, nonsmoking cancer patients tended to have an even lower P450IA2 activity than control nonsmokers, in

total contrast to P450IA activity. Current knowledge shows that P450IA2 activity has not been determined in lung cancer patients. Results from the present study show that there are large fundamental differences in the enzymes catalysing the caffeine and 1,7-DMX demethylation steps. Although P450IA2 activity is induced by cigarette smoking, both in this study and in those by Sesardic et al (1988) and Pantuck et al (1974), it does not appear to play an important role in the initiation of lung cancer. This may have something to do with the substrate specificity of the enzymes. P450IA2 preferentially metabolises arylamines (Thorgeirsson & Nebert, 1977; Kamataki et al, 1983; Hammons et al, 1985; Shimada & Okuda, 1988; Shimada et al, 1989; Butler et al, 1989a; Aoyama et al, 1989; Gonzalez et al, 1990) and hepatocarcinogenic aromatic amines are selective liver inducers of P450IA2 in the rat (Degawa et al, 1989). On the other hand, P450IA1 preferentially metabolises PAH (Kadlubar & Hammons, 1987). As both of these inducing compounds are present in cigarette smoke, one might expect P450IA2 activity to be higher in smokers than nonsmokers. While P450IA2 activates 4-aminobiphenyl to form haemoglobin-adducts in human lung tissue which correlate with the quantity of tobacco smoked, this enzyme does not appear to constitute an independent risk factor for lung cancer (Weston et al, 1991). It appears that PAH are more important in initiating lung cancer and may explain why P450IA2 activity is not altered in patients with this disease. As a large difference in the degree of 1,7-DMX demethylation (P450IA activity) in lung cancer patients has been found in this study, it seems very likely that P450IA1 is contributing to this pathway.

The current studies found no difference in P450IA or P450IA2 activities between the patients with carcinoma at different locations. P450IA activity was higher in patients with bronchial cancer and even the small group of 10 patients with carcinoma of the head and neck than that measured in the control population. AHH activity was found to be higher in patients with oral carcinoma (Trell et al, 1978; Trell & Korsgaard, 1978), pharyngeal cancer (Arnott et al, 1979) and laryngeal cancer (Trell et al, 1976) than healthy volunteers, which supports the findings in this study and is consistent with squamous cell cancer of the oral cavity being a smoking related cancer, in addition to squamous cell cancer of the lung (Trell & Korsgaard, 1978). AHH activity is high in patients with lung cancer, regardless of the position of the pulmonary tumour (Kouri et al, 1982).

Patients with tumors of squamous, adenocarcinoma and oat cell types were studied together in order to recruit a large number of subjects in accordance with Kellermann et al (1973b) and McLemore et al (1979). No significant differences in P450IA and P450IA2 activities existed between patients with tumors of different histological type, although one patient with a tumor of swan cell origin had the lowest P450IA activity. Therefore, for P450IA activity, the patients with squamous cancer and even the small group of patients with oat cell cancer had higher activities than the control population.

AHH activity was not significantly different in squamous and adenocarcinoma cell types by Sabadie et al (1981) and unaffected by histological type by Kouri et al (1982). Also, in one study where a quarter of the cancer population had adenocarcinoma,

patients with lung cancer were still found to have a much greater activity than healthy controls (Kellermann et al, 1973b). Kawajiri et al (1990) however, found that more of the patients with squamous cell lung cancer were of the rare CYPIA1 genotype corresponding to high AHH inducibility, than other cell types. Therefore, individuals with this rare genotype were at a 5-fold risk for squamous cell cancer, compared to a 3- fold risk for lung cancer of a different cell type.

One major point of concern while conducting this type of study is the relationship between hepatic P450IA activity determined by the caffeine test and levels of the same enzyme in lung. Test systems other than the lung have to be used in lung cancer patients because AHH activity is reduced in malignant tissue (Sabadie et al, 1981) although good correlations between AHH activities in lung and lymphocytes (McLemore et al, 1979) and human bronchus and colon and duodenum (Autrup & Harris, 1983) exist. Although human lung and respiratory nasal mucosa and olfactory tissue in rats (Foster et al, 1986) contain P450IA enzymes, the relationship between these and the hepatic P450IA enzymes is not yet clear, due to the tissue specificity that seems to characterise this P450 family.

Although the mean age of the cancer patients was greater than that for control volunteers, it is unlikely that high P450IA activity in lung cancer patients is a result of their advanced age, as no trend in increasing P450IA activity with age was shown to exist in healthy subjects. In fact, some investigators have reported that older patients have lower AHH activity and inducibility compared to young patients (Paigen et al, 1977;

Arnott et al, 1979). Kouri et al (1982) found that AHH activity was not affected by patient age nor by the presence of noncancerous, respiratory disorders, such as chronic obstructive airway disease. Therefore, it also seems unlikely that high P450IA activity in lung cancer patients is due to poor respiratory function. Patient gender is also unlikely to cause the high P450IA levels observed, as enzyme activity is unaffected by healthy subject gender in the current study in agreement with Arnott et al (1979). It would appear that high P450IA activity reflects inherent susceptibility of an individual to lung cancer. A genetic component controlling part of the process of lung cancer would not be unusual (Anderson, 1975; Mulvihill, 1975; Knudson, 1977; Schimke, 1978; Harris et al, 1980) and the disease occurs more frequently in the relatives of patients with lung cancer (Paigen et al, 1977).

As cancer of the lung is a disorder which remains silent during most of its pathologic evolution, its clinical onset is subtle and thus extremely difficult to diagnose early enough for effective therapy (Sanderson & Jett, 1989). Important measures should be taken to detect individuals with high P450IA activity at an early stage as this study shows that there is both an increased frequency of high P450IA activity in nonsmoking cancer patients and high P450IA inducibility in smoking cancer patients. Therefore, P450IA but not P450IA2 activity, as determined by the caffeine test may be of extreme value as a marker for detection of susceptibility to carcinoma of the lung, head and neck, by virtue of its possible genetic polymorphic status coupled with its ability to metabolise certain chemical carcinogens.

5.3 P450IA AND P450IA2 ACTIVITIES IN PATIENTS WITH LEUKAEMIA

5.3.1 Introduction

Large interindividual differences in P450IA and IA2 activities exist in a group of healthy subjects. Subjects with high P450IA activity seem to be predisposed for lung cancer, which supports observations in the murine model that high AHH inducibility leads to increased susceptibility for lung carcinoma. In contrast, mice with low AHH inducibility are at increased risk for leukaemia and aplastic anaemia. Thus the differential susceptibility of the human population to chemical carcinogens in various tissues may be partially attributed to individual differences in the degree of carcinogen metabolism.

By giving caffeine as a probe drug to a group of patients with leukaemia and measuring the caffeine metabolites of interest in urine, P450IA and P450IA2 activities were determined and compared to those from a control population (Section 2.3.2).

5.3.2 Results

The enzyme activities for the patients with leukaemia are shown in Table 5.3.1. When compared to control volunteers, P450IA (Fig.5.3.1) and P450IA2 (Fig.5.3.2) activities in patients with leukaemia were not significantly different ($p>0.3$). The mean age of 54 patients with leukaemia was 61.85 $\pm$ 2.13 y. This was significantly higher than the mean age of 40.24 $\pm$ 0.95 y for 277 control volunteers ($p<0.001$).

5.3.3 Effect of Cigarette Smoke

P450IA and P450IA2 activities were studied in 54 patients with leukaemia of known smoking status; 7 were smokers and 47 were

Table 5.3.1 - P450IA and P450IA2 activities in patients with leukaemia and control volunteers

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Control	277	11.64	14.52 ± 0.87	1.15 -130.33
	Leukaemia	54	13.13	15.81 ± 1.40	1.91 - 46.99
P450IA2	Control	254	74.68	97.29 ± 5.45	7.92 -607.62
	Leukaemia	54	68.42	85.81 ± 8.78	5.50 -294.03

nonsmokers. The results are shown in Table 5.3.2.

Table 5.3.2 - P450IA and P450IA2 activities in smoking and nonsmoking leukaemia patients

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Smokers	7	10.70	15.97 ± 4.09	5.22 - 33.24
	Nonsmokers	47	13.55	15.79 ± 1.51	1.91 - 46.99
P450IA2	Smokers	7	76.88	99.37 ±35.41	11.58 - 294.03
	Nonsmokers	47	64.13	83.79 ± 8.78	5.50 - 260.86

Although P450IA and P450IA2 activities tended to be higher in smoking patients with leukaemia than nonsmokers, the results were not significantly different ($p>0.5$).

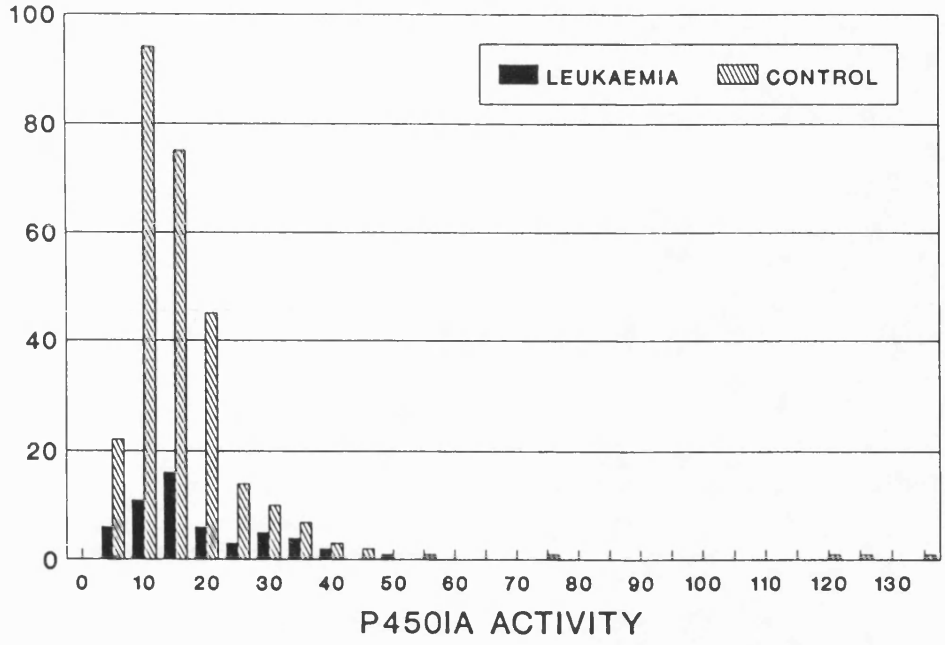


Figure 5.3.1 - P450IA activity in patients with leukaemia, compared to controls.

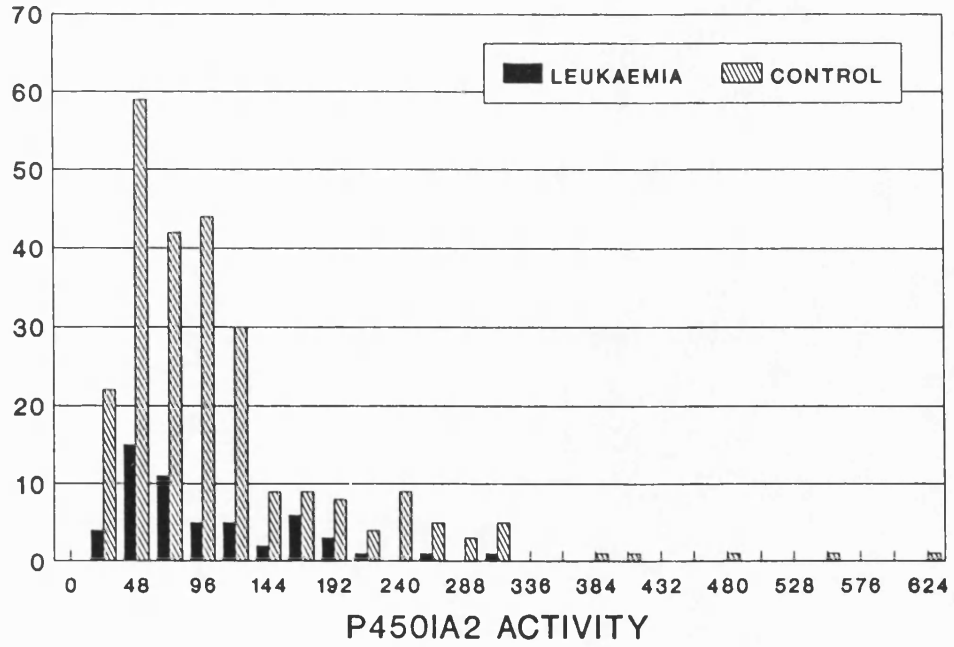


Figure 5.3.2 - P450IA2 activity in patients with leukaemia, compared to controls.

In order to compare any difference in enzyme activities between healthy subjects and patients with leukaemia further, smokers and nonsmokers from each population were also compared separately. The results are shown in Table 5.3.3.

Table 5.3.3 - P450IA and P450IA2 activities in smoking and nonsmoking patients with leukaemia and control volunteers

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Smokers				
	-control	70	13.76	16.55 ± 1.94	1.15 - 118.46
	-leukaemia	7	10.70	15.97 ± 4.09	5.22 - 33.24
	Nonsmoker				
	-control	185	10.09	12.21 ± 0.57	2.95 - 54.89
	-leukaemia	47	13.55	15.79 ± 4.09	5.22 - 33.24
P450IA2	Smokers				
	-control	70	85.48	118.66 ±12.49	7.92 - 607.62
	-leukaemia	7	76.88	99.37 ±35.41	11.58 - 294.03
	Nonsmokers				
	-control	184	68.90	89.49 ± 5.74	11.58 - 550.23
	-leukaemia	47	64.13	83.79 ± 8.78	5.50 - 260.86

P450IA and P450IA2 activities in smoking leukaemia patients were not significantly different than that of smoking control subjects ($p>0.5$).

When the nonsmoking population was studied, it was found that P450IA and P450IA2 activities were not significantly different

in nonsmoking patients with leukaemia and control nonsmokers ($p>0.05$).

5.3.4 Effect of Leukaemia Type

P450IA and P450IA2 activities were studied in 37 patients with leukaemia of known type. 29 patients had chronic and 8 had acute leukaemia. The enzyme activities for each group are shown in Table 5.3.4.

Table 5.3.4 - P450IA and P450IA2 activities in patients with chronic and acute leukaemia

Enzyme	Group	n	Median	Mean activity ± SEM	Range
P450IA	Chronic	29	14.91	18.47 ± 2.15	1.91 - 46.99
	Acute	8	10.87	14.47 ± 3.45	5.48 - 32.29
P450IA2	Chronic	29	71.29	93.11 ±12.66	9.95 - 294.03
	Acute	8	71.63	89.10 ±19.57	27.07 - 183.06

P450IA and P450IA2 activities were not significantly different in patients with chronic or acute leukaemia ($p>0.5$) and similar to those determined in the control population.

5.3.5 Genetic Control

P450IA Activity

The frequency distribution histogram for P450IA activity in 54 patients with leukaemia is depicted in Fig.5.3.3. The summary statistics for this distribution are shown in Table 5.3.5.

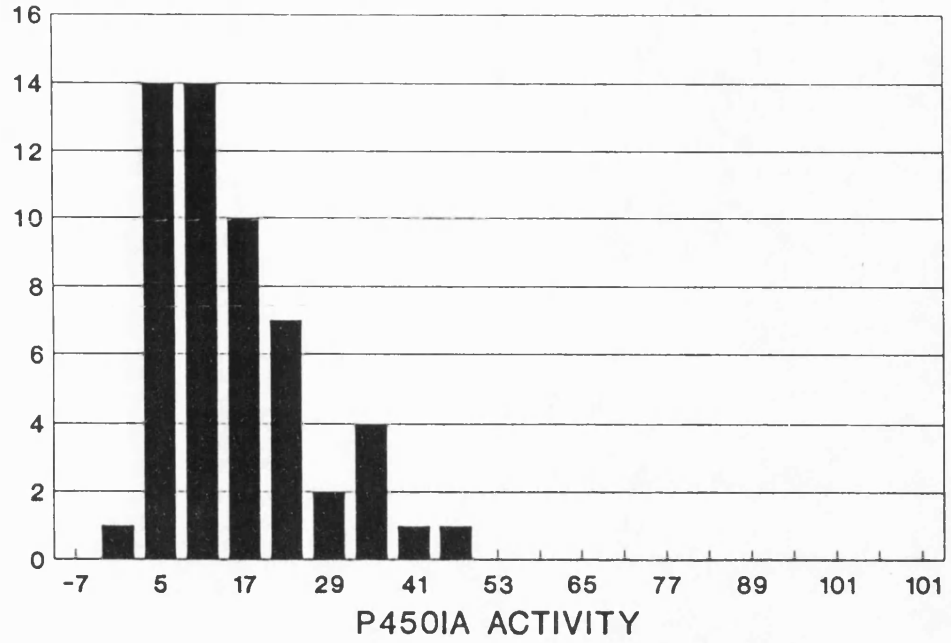


Figure 5.3.3 - Frequency distribution histogram of P450IA activity in 54 patients with leukaemia.

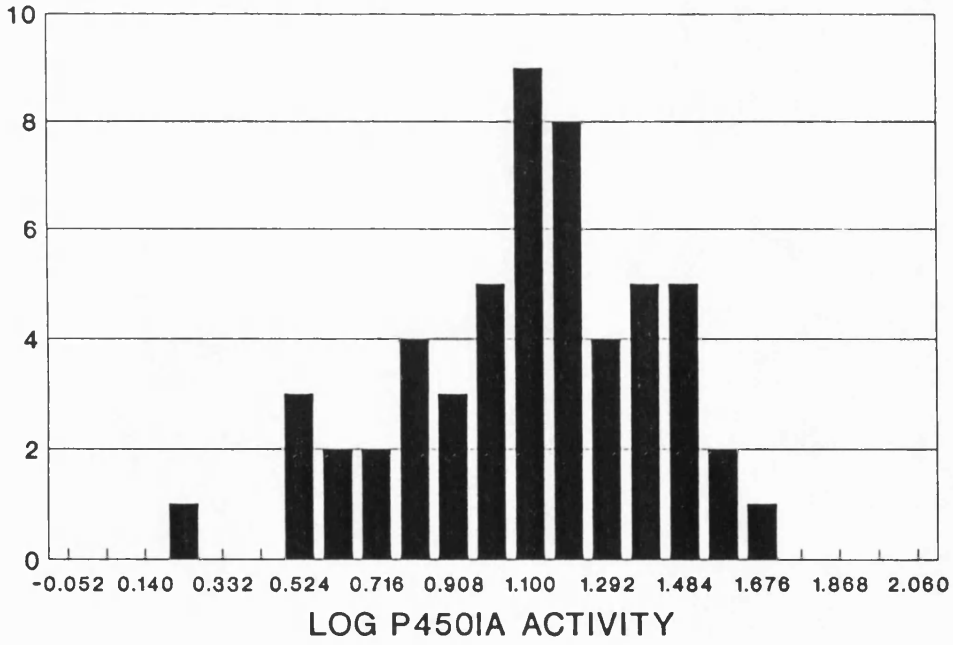


Figure 5.3.4 - Log frequency distribution histogram of P450IA activity in 54 patients with leukaemia.

Table 5.3.5 - Summary Statistics for P450IA activity in 54 patients with leukaemia

Median	13.13
Mean	15.81
Standard error	1.40
Standard deviation	10.30
Minimum	1.91
Maximum	46.99
Skewness	0.99
Standardized skewness	2.96
Kurtosis	0.50
Standardized kurtosis	0.76

25-fold variation in P450IA actviity exists in 54 patients. It can be seen from the histogram and Table 5.3.5 that the distribution is positively skewed and shows a small degree of kurtosis. Statistical tests show that the data is significantly different from normal and possibly bimodal (Chi-square test, $p<0.05$; Lilly-Fors test, $p<0.01$).

As the data show a strong degree of skewness and kurtosis, the data were logged and re-examined in order to detect bimodality of the distribution. The frequency distribution histogram for log P450IA activity in 54 patients with leukaemia is shown in Fig.5.3.4.

The logged distribution is slightly positively skewed statistical tests performed on the data suggested that P450IA activity was unimodal.

The proportion of leukaemia patients with high P450IA activity (ratio >26) is shown in Table 5.3.6.

Table 5.3.6 - The proportion of leukaemia patients with high P450IA activity

Group	P450IA activity			
	High	%	Low	%
Leukaemia	8/54	(14.8%)	46/54	(85.2%)
-Smokers	2/ 7	(28.6%)	5/ 7	(71.4%)
-Nonsmokers	6/47	(12.8%)	41/47	(87.2%)
Control	25/277	(9.0%)	252/277	(91.0%)
-Smokers	8/ 70	(11.4%)	62/ 70	(88.6%)
-Nonsmokers	12/185	(6.5%)	173/185	(93.5%)

The proportion of leukaemia patients with high P450IA activity was 14.8% (Fig.5.3.3). This was not significantly different to the proportion (9%) of control volunteers with high P450IA activity ($p>0.05$). The proportion of smoking leukaemia patients (28.6%) with high P450IA activity was not significantly different to that in control smokers (11.4%) ($p>0.05$). The proportion of nonsmoking leukaemia patients (12.8%) with high P450IA activity was also not significantly different to that of control nonsmokers (6.5%) ($p>0.05$). The proportion of smoking and nonsmoking leukaemia patients with high P450IA activity was not significantly different ($p>0.05$).

P450IA2 Activity

The frequency distribution histogram for P450IA2 activity in 54 patients with leukaemia is depicted in Fig.5.3.5. The summary statistics for this distribution are shown in Table 5.3.7.

Table 5.3.7 - Summary Statistics for P450IA2 activity in 54 patients with leukaemia

Median	68.42
Mean	85.81
Standard error	8.78
Standard deviation	64.54
Minimum	5.50
Maximum	294.03
Skewness	1.17
Standardized skewness	3.52
Kurtosis	1.20
Standardized kurtosis	1.80

53-fold variation in P450IA2 activity exists in 54 patients. It can be seen from the histogram and Table 5.3.6 that the distribution is strongly positively skewed and shows a degree of kurtosis. The standardized coefficients indicate that the data may deviate significantly from normality which is confirmed by statistical tests (Chi-square test, $p<0.005$; Lilly-Fors test, $p<0.01$).

As the data show a strong degree of skewness and kurtosis, the data were logged and re-examined in order to detect bimodality

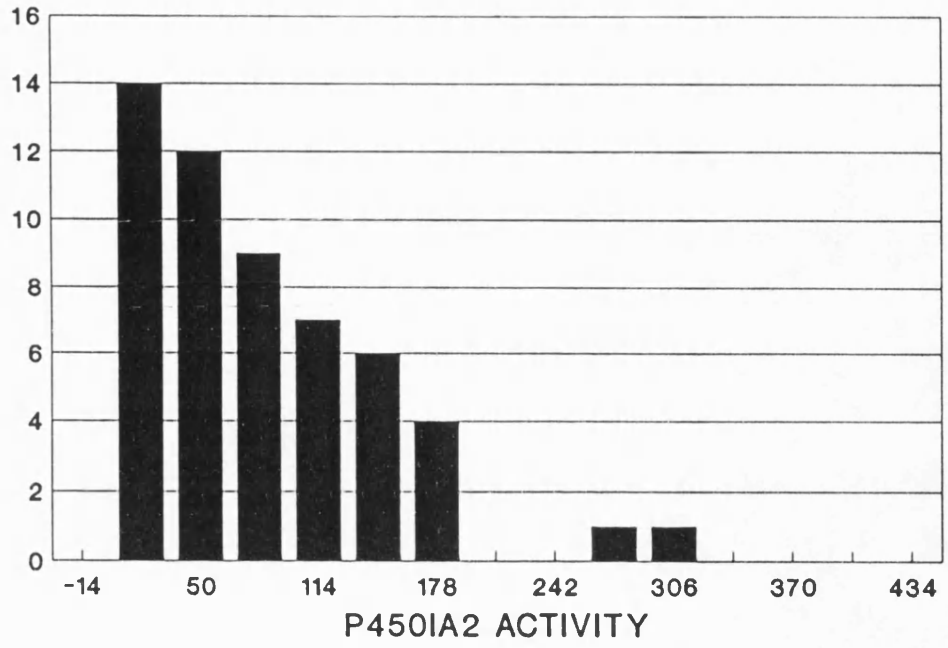


Figure 5.3.5 - Frequency distribution histogram of P450IA2 activity in 54 patients with leukaemia.

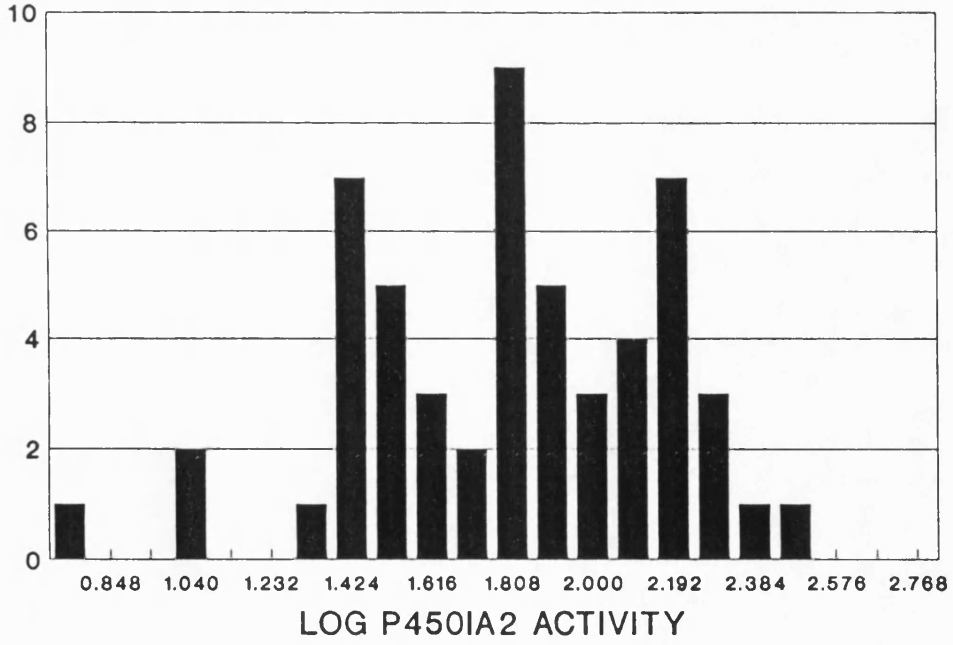


Figure 5.3.6 - Log frequency distribution histogram of P450IA2 activity in 54 patients with leukaemia.

of the distribution. The frequency distribution histogram for log P450IA2 activity in 54 patients with leukaemia is shown in Fig.5.3.6. The distribution is slightly positively skewed but the the data is likely to come from a normal distribution as statistical tests performed on the data suggested that P450IA2 activity in leukaemia patients was unimodal.

The proportion of leukaemia patients with high P450IA2 activity (ratio >162) is shown in Table 5.3.8.

Table 5.3.8 - The proportion of leukaemia patients with high P450IA2 activity

Group	P450IA2 activity			
	High	%	Low	%
Leukaemia	6/54	(11.1%)	48/54	(88.9%)
-Smokers	1/ 7	(14.3%)	6/ 7	(85.7%)
-Nonsmokers	5/47	(10.6%)	42/47	(89.4%)
Control	42/254	(16.5%)	212/254	(83.5%)
-Smokers	19/ 70	(27.1%)	51/ 70	(72.9%)
-Nonsmokers	23/184	(12.5%)	161/184	(87.5%)

The proportion of leukaemia patients with high P450IA2 activity was 11.1% (Fig.5.3.5). This was not significantly different to the proportion (16.5%) of control subjects with high P450IA2 activity ($p>0.05$). The proportion of smoking leukaemia patients (14.3%) with high P450IA2 activity was not significantly different to that in control smokers (27.1%) ($p>0.05$). The

proportion of nonsmoking leukaemia patients (10.6%) with high P450IA2 activity was also not significantly different to that of control nonsmokers (12.5%; $p>0.05$). The proportion of smoking and nonsmoking leukaemia patients with high P450IA2 activity was not significantly different ($p>0.05$).

5.3.6 Discussion

P450IA activity determined in 54 patients with leukaemia did not differ significantly from that measured in 277 control volunteers. Results from the present study would therefore suggest that P450IA activity is not a contributing factor in leukaemia development.

Several studies have shown that mice with high AHH inducibility (Ah responsive) are at increased risk for PAH induced pulmonary tumors and solid tumors at the site of PAH inoculation (Kouri, 1976; Kouri *et al*, 1980; Nebert, 1980b) whereas mice with low AHH inducibility had increased susceptibility for oral drug-induced leukaemia and aplastic anaemia (Nebert *et al*, 1977; Duran-Reynals *et al*, 1978; Nebert & Jensen, 1979; Nebert *et al*, 1980b). As less P450IA induction occurs throughout the body in nonresponsive mice, less chemical is metabolised into active metabolites in lung and skin but more of the chemical reaches distant tissues, including the bone marrow, where it may be activated and bind to DNA (Nebert, 1981b). The nonresponsive mouse is therefore more prone to chemically induced lymphoma, leukaemia and bone marrow toxicity (Nebert, *et al*, 1980b).

Support for the murine model occurring in man came from a study on 37 patients with acute leukaemia of childhood (Blumer *et al*, 1979). A greater proportion of these patients were of the low

inducibility phenotype, compared to healthy subjects. Results from the present study show that smoking leukaemia patients have similar P450IA activity to nonsmoking patients, unlike lung cancer patients. It is possible therefore that P450IA is not induced in leukaemia patients to the same extent as it is in patients with other types of cancer, in support of Blumer et al (1979). Results from this study do not support the theory of low P450IA activity causing increased susceptibility for leukaemia, as P450IA activity in the patients was similar to that measured in healthy subjects, in agreement with Levine et al (1984). However, unlike the study by Kellermann (1973a) where a trimodal distribution was obtained, the distribution for P450IA activity in this study appeared to be bimodal, such that subjects with homozygous low P450IA activity could not be identified. Therefore, although the proportion of leukaemia patients with high P450IA activity was not significantly different from that in control subjects, we cannot rule out the possibility that the proportion of patients with low activity may be different from that in control volunteers.

It seems unlikely that smoking status was responsible for the lack of low P450IA activity in these patients, as smokers had a similar enzyme activity to nonsmokers in this study.

P450IA2 activity was also not significantly different in patients with leukaemia compared to the control population. While P450IA1 has been detected in bone marrow tissue (Schnier et al, 1989), it is not thought that P450IA2 activity is induced in such tissue. It seems unlikely therefore that differences in P450IA2 activity will be important in leukaemia initiation.

5.4 P450IA AND P450IA2 ACTIVITIES IN PATIENTS WITH COLORECTAL CARCINOMA AND FAMILIAL ADENOMATOUS POLYPOSIS

5.4.1 Introduction

P450IA2 activity appears to be expressed in all human liver samples tested, unlike P450IA1 (Wrighton et al, 1986). P450IA2 has high specificity for the N-hydroxylation of a number of aromatic amines (Kadlubar & Hammons, 1987). For primary arylamines, metabolic N-oxidation by hepatic cytochrome P450IA2 is generally regarded as the initial activation step leading to carcinogenesis (Nebert et al, 1987; Hammons et al, 1985; Butler et al, 1989a,b) and in humans, P450IA2 appears to be primarily responsible for the mutagenic activation of several heterocyclic amines (Shimada et al, 1989; Battula et al, 1990). Since we have shown that considerable interindividual variability in this enzyme activity exists in human populations, it is possible that such variation, due to environmental and/or genetic factors, may contribute to individual differences in susceptibility to arylamine-induced cancers.

By giving caffeine as a probe drug to a group of patients with familial adenomatous polyposis and colorectal carcinoma, P450IA and P450IA2 activities were determined and compared to those of a control population and a group of relatives at risk for developing FAP (Section 2.3.2).

5.4.2 Results

P450IA activities for the patients with FAP and colorectal carcinoma are shown in Table 5.4.1. P450IA activities in patients with FAP ($p>0.05$) (Fig.5.4.1) and their relatives at risk for FAP ($p>0.5$) (Fig.5.4.2) were not significantly

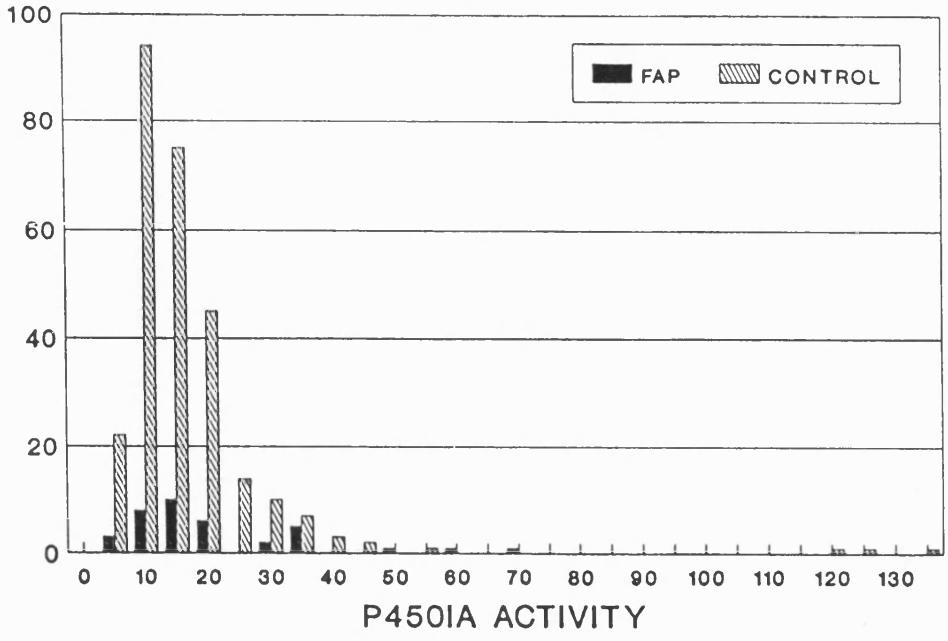


Figure 5.4.1 - P450IA activity in patients with FAP, compared to controls.

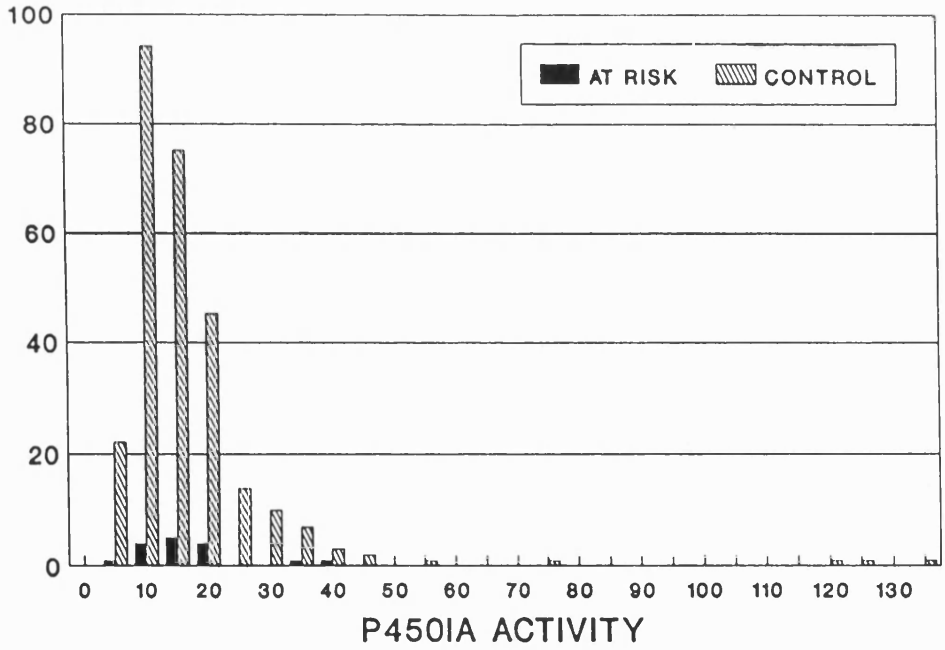


Figure 5.4.2 - P450IA activity in relatives at risk for FAP, compared to controls.

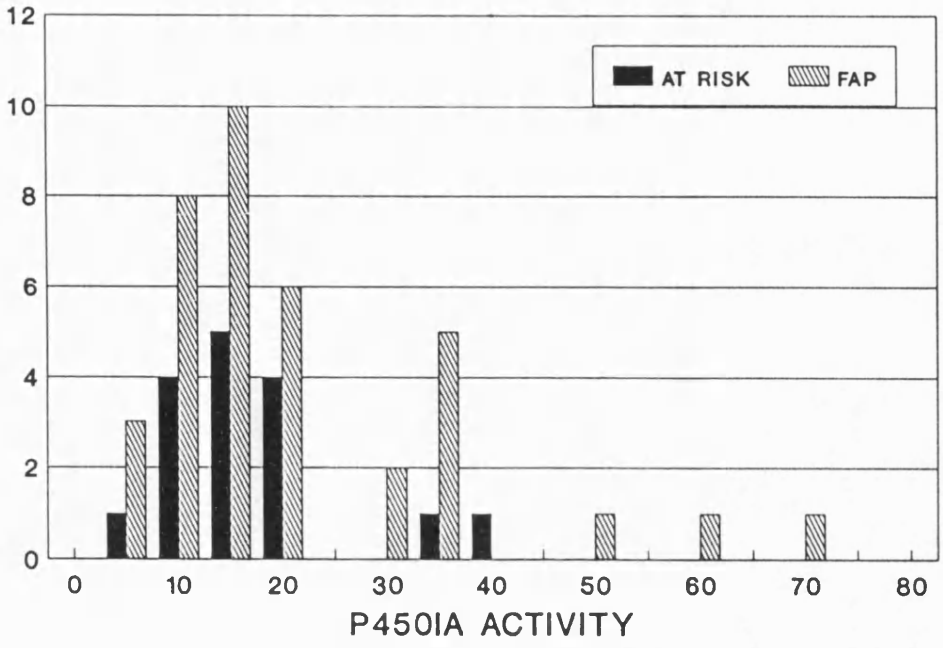


Figure 5.4.3 - P450IA activity in patients with FAP, compared to their relatives at risk for FAP.

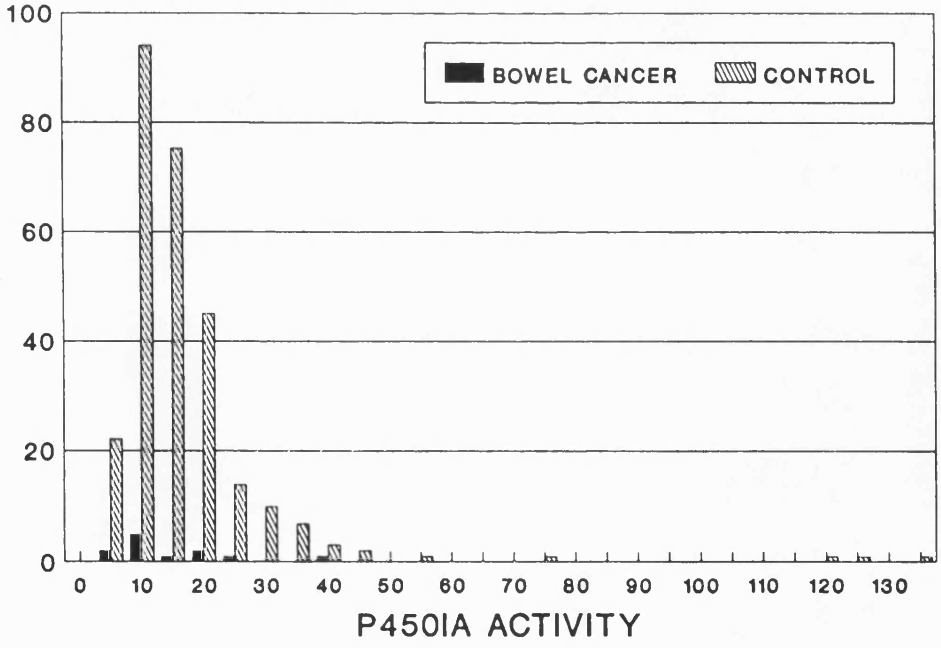


Figure 5.4.4 - P450IA activity in patients with colorectal carcinoma, compared to controls.

Table 5.4.1 - P450IA activities in patients with colorectal carcinoma and FAP

Group	n	Median	Mean activity ± SEM	Range
Control	277	11.64	14.52 ± 0.87	1.15 -130.33
Colorectal	12	8.83	12.97 ± 2.91	4.55 - 38.76
FAP	37	14.04	18.37 ± 2.43	3.40 - 65.60
At Risk	16	10.85	14.74 ± 2.37	4.23 - 39.72

different from those determined in control subjects. P450IA activity was not significantly different between FAP patients and the group at risk for FAP ($p>0.5$) (Fig.5.4.3). Patients with bowel cancer had similar P450IA activities to the control population ($p>0.5$) (Fig.5.4.4).

P450IA2 activities for patients with colorectal carcinoma, FAP and their relatives at risk for FAP are shown in Table 5.4.2. P450IA2 activity was significantly greater in patients with FAP ($p<0.005$) (Fig.5.4.5) and in their relatives ($p<0.05$) (Fig.5.4.6) than control volunteers. The mean enzyme activity in FAP patients was not significantly different from that in the group at risk for FAP ($p>0.5$) (Fig.5.4.7). In contrast, P450IA2 activity in the patients with colorectal carcinoma was not significantly different from that of control subjects ($p>0.1$) (Fig.5.4.8).

The mean age of 37 patients with FAP was 36.38 ± 2.44 y. This was not significantly different from the mean age of 40.24 ±

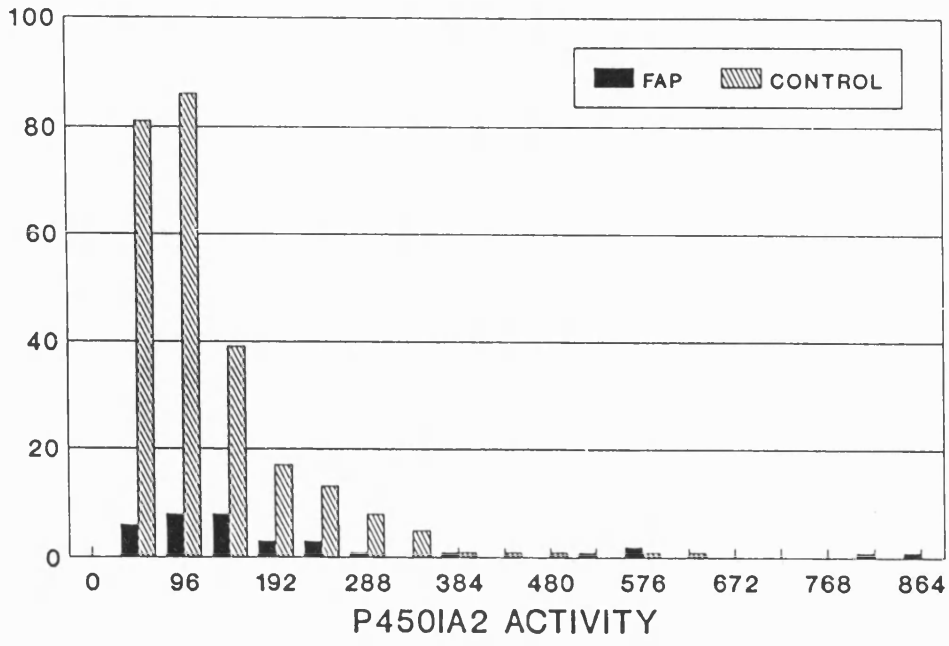


Figure 5.4.5 - P450IA2 activity in patients with FAP, compared to controls.

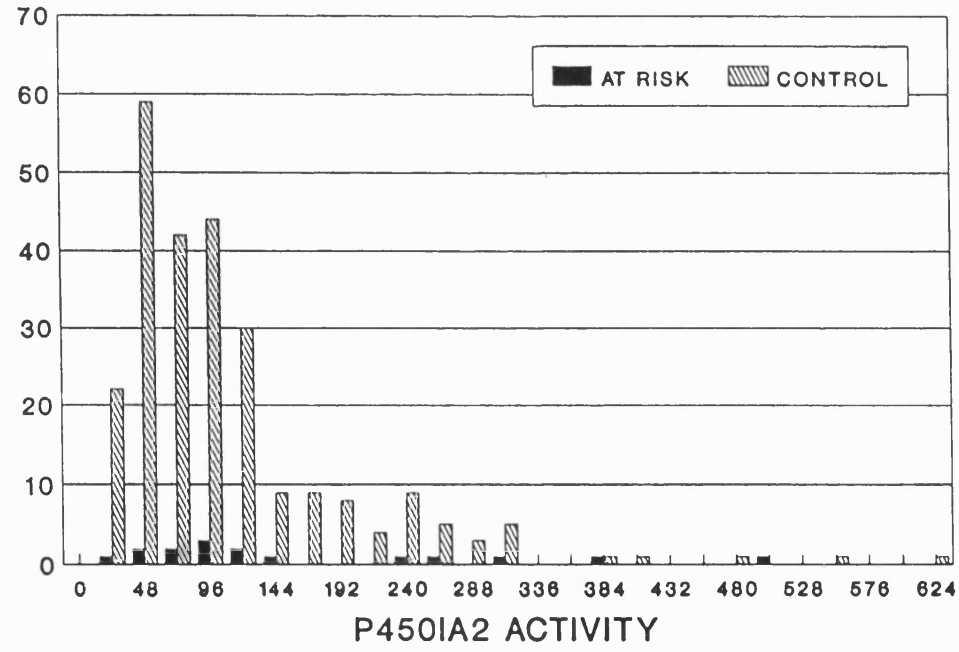


Figure 5.4.6 - P450IA2 activity in relatives at risk for FAP, compared to controls.

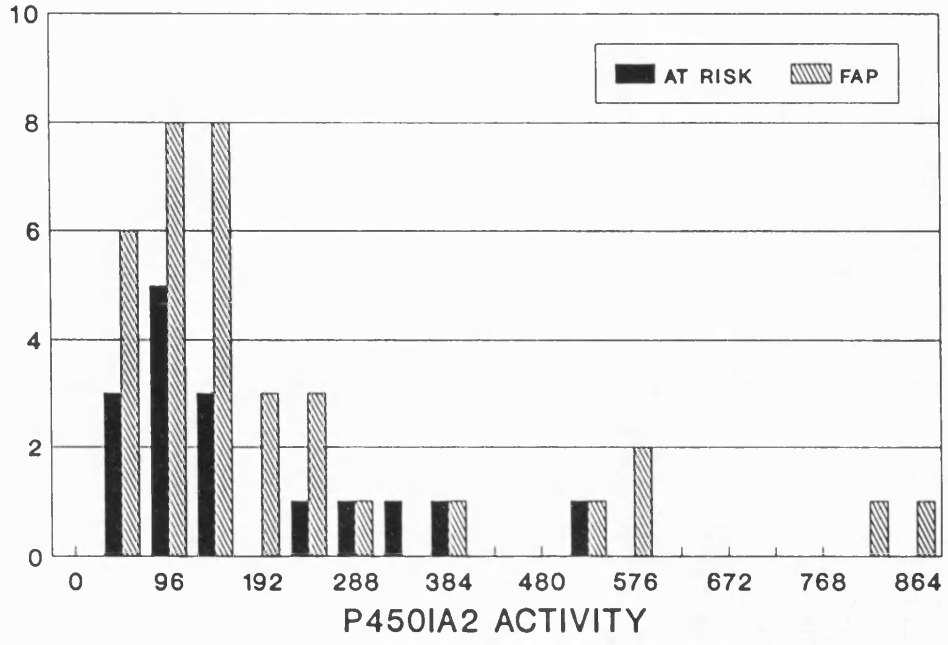


Figure 5.4.7 - P450IA2 activity in patients with FAP, compared to their relatives at risk for FAP.

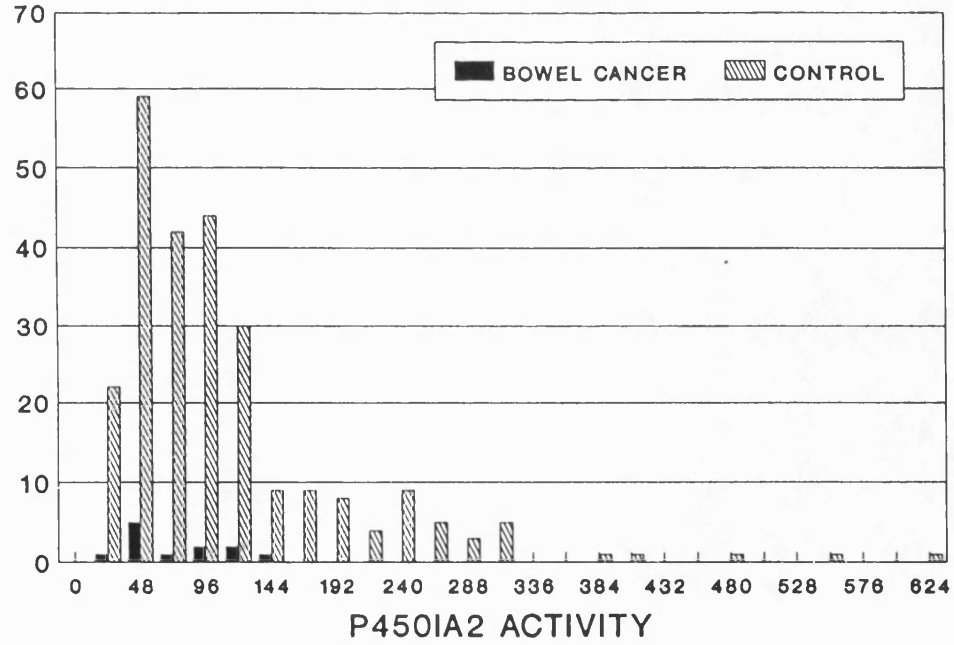


Figure 5.4.8 - P450IA2 activity in patients with colorectal carcinoma compared to controls.

Table 5.4.2 - P450IA2 activities in patients with colorectal carcinoma and FAP

Group	n	Median	Mean activity ± SEM	Range
Control	254	74.68	97.29 ± 5.45	7.92 - 607.62
Colorectal	12	51.88	64.76 ± 10.60	23.89 - 129.94
FAP	35	108.21	190.02 ± 35.85	6.25 - 846.67
At Risk	16	104.02	157.04 ± 34.58	18.04 - 502.45

0.95 y for 277 control volunteers ($p>0.1$). The mean age of 16 of their relatives at risk for polyposis was 31.25 ± 4.06 y, which was not significantly different from the mean age of the FAP patients ($p>0.1$), but significantly less than the mean age of the control volunteers ($p<0.05$). The mean age of the patients with colorectal carcinoma was 70 ± 2.8 y. This was significantly greater than the mean age of the control volunteers ($p<0.001$).

5.4.3 Effect of Cigarette Smoke

P450IA activity was studied in 5 smokers and 27 nonsmokers with FAP and in 6 smoking and 10 nonsmoking relatives at risk for FAP. None of the patients with colorectal carcinoma were smokers. The results are shown in Table 5.4.3.

P450IA activity tended to be higher in smoking patients with FAP than nonsmoking patients ($p>0.1$). This was also the case for the relatives at risk for FAP ($p>0.1$).

P450IA2 activity was studied in 5 smokers and 25 nonsmokers with

Table 5.4.3 - P450IA activity in smoking and nonsmoking patients with FAP and their relatives at risk for FAP.

Group		n	Median	Mean activity ± SEM	Range
FAP	Smokers	5	19.86	26.53 ± 8.20	8.85 - 55.08
	Nonsmokers	27	12.11	16.78 ± 2.78	3.40 - 65.60
At Risk	Smokers	6	14.38	17.42 ± 4.89	7.89 - 39.72
	Nonsmokers	10	10.84	13.13 ± 2.50	4.23 - 31.02

FAP and in 6 smoking and 10 nonsmoking relatives at risk for FAP. The results are shown in Table 5.4.4.

Table 5.4.4 - P450IA2 activity in smoking and nonsmoking patients with FAP and their relatives at risk for FAP

Group		n	Median	Mean activity ± SEM	Range
FAP	Smokers	5	543.69	454.86 ±122.68	99.30 - 802.84
	Nonsmokers	25	103.31	152.86 ± 36.27	6.25 - 846.67
At Risk	Smokers	6	177.11	216.40 ± 64.30	64.16 - 502.45
	Nonsmokers	10	78.30	121.43 ± 37.87	18.05 - 380.15

P450IA2 activity was significantly greater in smoking patients

with FAP than nonsmoking patients ($p<0.01$) and tended to be higher in smoking relatives at risk for FAP than in nonsmoking relatives ($p=0.01$). Due to the increased P450IA2 activity in patients with FAP and relatives at risk for FAP, smokers and nonsmokers were studied separately and compared to control subjects. The results are shown in Table 5.4.5.

Table 5.4.5 - P450IA2 activity in smoking and nonsmoking FAP patients and healthy subjects

Group		n	Median	Mean activity ± SEM	Range
Smokers	-Control	70	85.48	118.66 ± 12.49	7.92 -607.62
	-FAP	5	543.69	454.86 ±122.68	99.30 -802.84
	-At Risk	6	177.11	216.40 ± 64.30	64.16 -502.45
Nonsmokers	-Control	184	68.90	89.49 ± 5.74	11.58 -550.23
	-FAP	25	103.31	152.86 ± 36.27	6.25 -846.67
	-At Risk	10	78.30	121.43 ± 37.87	18.05 -380.15

5 smoking patients with FAP had a significantly higher P450IA2 activity than control smokers ($p<0.001$). 6 smoking relatives at risk for FAP also tended to have higher P450IA2 activity than control smokers ($p=0.052$). P450IA2 activity in smoking FAP patients was not significantly different from smoking relatives at risk for the disease ($p>0.1$).

The nonsmoking patients with FAP had a significantly higher P450IA2 activity than control nonsmokers ($p<0.05$). P450IA2

activity in the control group of nonsmoking relatives was not significantly different from control nonsmokers ($p>0.1$). P450IA2 activity in nonsmoking FAP patients was not significantly different to that of their relatives at risk for FAP ($p>0.5$).
As the patients with large bowel cancer were all nonsmokers, they were compared to control nonsmokers. P450IA2 activity was not significantly different between these two groups ($p>0.1$).

5.4.4 Genetic Control

P450IA Activity

The frequency distribution histogram for P450IA activity in 37 patients with FAP is depicted in Fig.5.4.9. The summary statistics for this distribution are shown in Table 5.4.6.

Table 5.4.6 - Summary Statistics for P450IA activity in 37 patients with FAP

Median	14.04
Mean	18.37
Standard error	2.43
Standard deviation	14.78
Minimum	3.40
Maximum	65.60
Skewness	1.60
Standardized skewness	3.96
Kurtosis	2.42
Standardized kurtosis	3.00

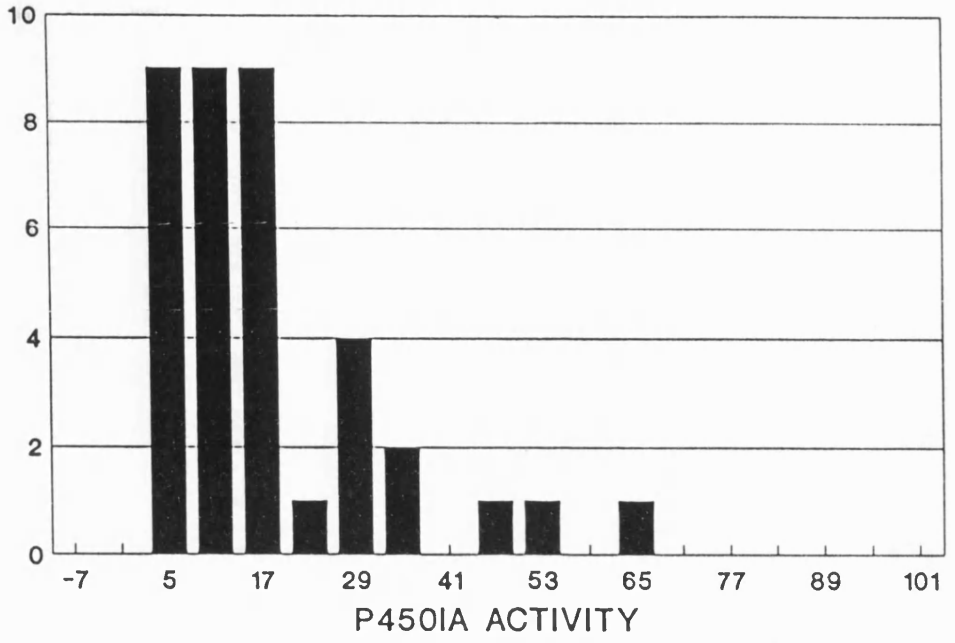


Figure 5.4.9 - Frequency distribution histogram of P450IA activity in 37 patients with FAP.

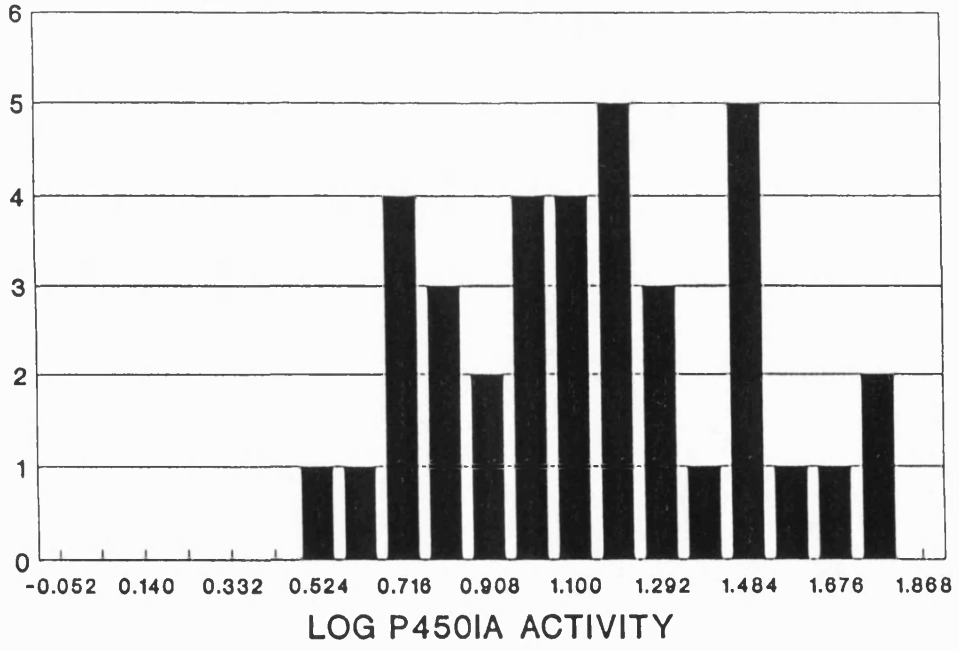


Figure 5.4.10 - Log frequency distribution histogram of P450IA activity in 37 patients with FAP.

19-fold variation in P450IA activity exists in FAP patients. It can be seen from the histogram and Table 5.4.6 that the distribution is strongly positively skewed and shows a large degree of kurtosis. The Chi-square test, but not the K-S test, is significantly different from normal ($p<0.0005$) and suggests that the data deviates from normality. In addition the Lilly-Fors test indicated that P450IA activity was highly significantly different from those expected for a normal distribution ($p<0.01$). However, the logged data were found to be not significantly different from normal (Fig.5.4.10).

The frequency distribution histogram for P450IA activity in 16 relatives at risk for FAP is depicted in Fig.5.4.11. The summary statistics for this distribution are shown in Table 5.4.7.

Table 5.4.7 - Summary Statistics for P450IA activity in 16 relatives at risk for FAP

Median	10.85
Mean	14.74
Standard error	2.37
Standard deviation	9.49
Minimum	4.23
Maximum	39.72
Skewness	1.53
Standardized skewness	2.50
Kurtosis	2.28
Standardized kurtosis	1.86

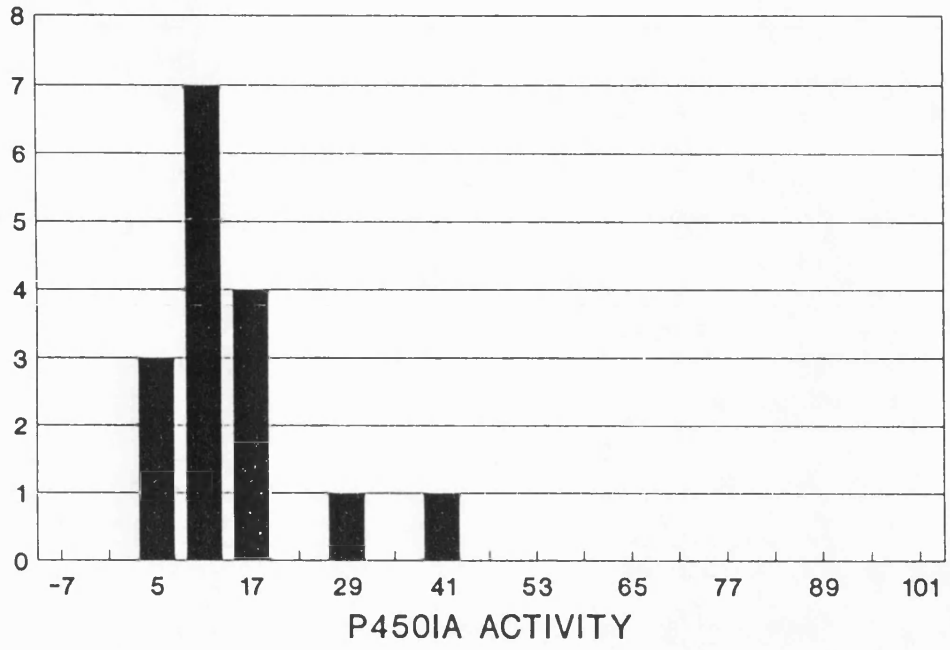


Figure 5.4.11 - Frequency distribution histogram of P450IA activity in 16 relatives at risk for FAP.

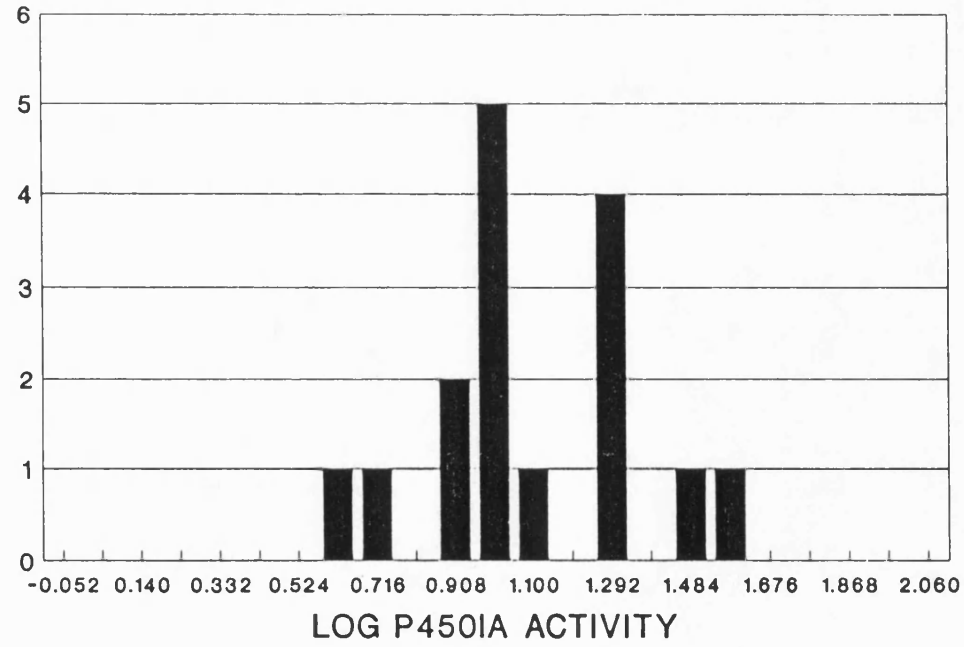


Figure 5.4.12 - Log frequency distribution histogram of P450IA activity in 16 relatives at risk for FAP.

9-fold variation in P450IA activity existed. It can be seen from the histogram and Table 5.4.7 that the distribution is strongly positively skewed and shows a degree of kurtosis. The frequency distribution histogram for log P450IA activity in the 16 subjects is shown in Fig.5.4.12. All of the statistical tests performed on the data suggested that P450IA activity was unimodal in these subjects.

The proportion of FAP patients and their relatives at risk for FAP with high P450IA activity (ratio >26) is shown in Table 5.4.8.

Table 5.4.8 - The proportion of FAP patients and relatives at risk for FAP with high P450IA activity

Group	P450IA activity			
	High	%	Low	%
FAP	9/37	(24.3%)	28/37	(75.7%)
-Smokers	2/ 5	(40.0%)	3/ 5	(60.0%)
-Nonsmokers	5/27	(18.5%)	22/27	(81.5%)
At Risk	2/16	(12.5%)	14/16	(87.5%)
-Smokers	1/ 6	(16.7%)	5/ 6	(83.3%)
-Nonsmokers	1/10	(10.0%)	9/10	(90.0%)
Control	25/277	(9.0%)	252/277	(91.0%)
-Smokers	8/ 70	(11.4%)	62/ 70	(88.6%)
-Nonsmokers	12/185	(6.5%)	173/185	(93.5%)

24.3% of FAP patients had high P450IA activity (Fig.5.4.9). This was significantly greater than the proportion (9%) of control volunteers with high P450IA activity ($p<0.01$). The proportion of smoking FAP patients with high P450IA activity (40%) tended to be greater than the same proportion of control smokers (11.4%). The number of nonsmoking FAP patients with high P450IA activity (18.5%) was significantly greater than the control nonsmokers with a high enzyme activity (6.5%) ($p<0.05$). The number of smoking FAP patients with high P450IA activity was not significantly different from that in nonsmokers ($p>0.05$).
The proportion of relatives at risk with high P450IA activity (12.5%) (Fig.5.4.11) was not significantly different from that in the control population (9%) ($p>0.05$) or in the patients with FAP (24.3%) ($p>0.05$). The proportion of smoking or nonsmoking relatives with high P450IA activity was not significantly different to the proportion of control smokers and nonsmokers with high P450IA activity ($p>0.05$). The number of smoking relatives at risk for FAP with high P450IA activity was also not significantly different from that in nonsmokers ($p>0.05$).

The frequency distribution histogram for P450IA activity in 12 patients diagnosed with colorectal cancer is depicted in Fig.5.4.13. The summary statistics for this distribution are shown in Table 5.4.9. 9-fold variation in P450IA activity existed. It can be seen from the histogram and Table 5.4.9 that the distribution is positively skewed, although the K-S test and the Lilly-Fors test indicate that it is unlikely that the data for this distribution deviates from normality. The log frequency distribution for P450IA activity in the 12 colorectal cancer

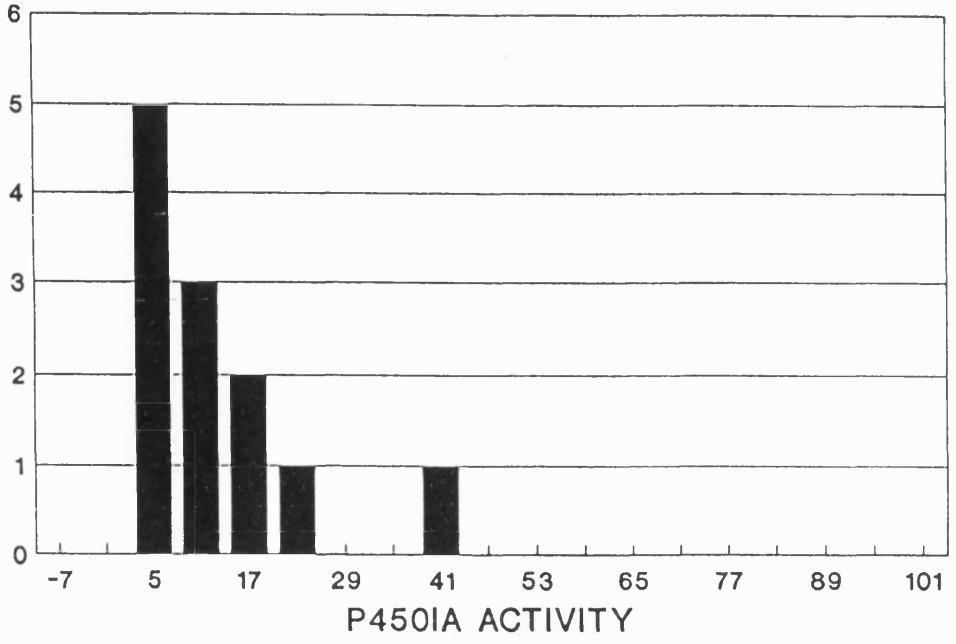


Figure 5.4.13 - Frequency distribution histogram of P450IA activity in 12 patients with colorectal cancer.

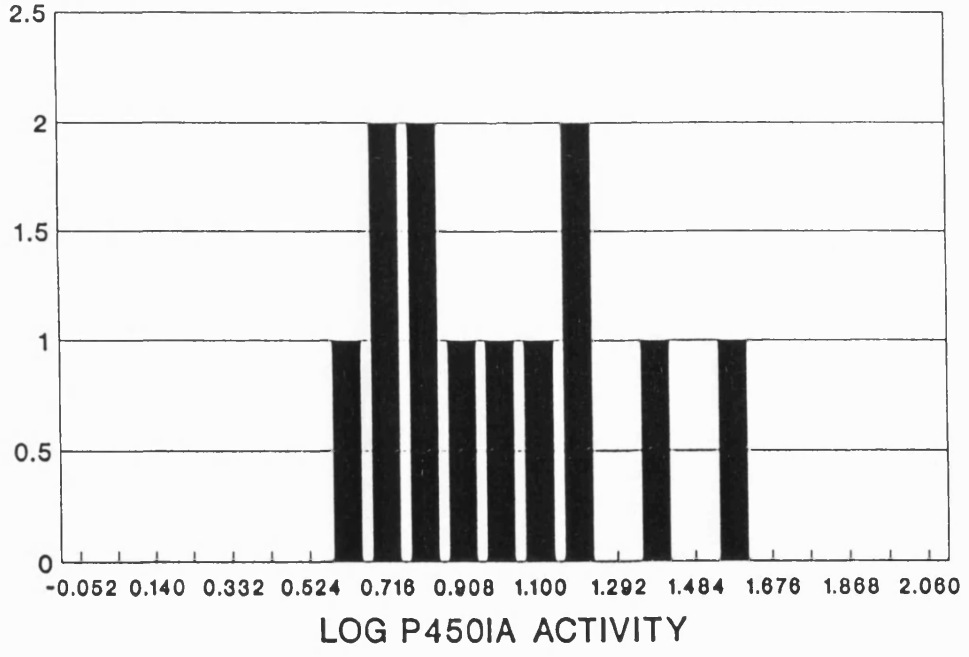


Figure 5.4.14 - Log frequency distribution histogram of P450IA activity in 12 patients with colorectal cancer.

Table 5.4.9 - Summary Statistics for P450IA activity in 12 patients with colorectal cancer

Median	8.83
Mean	12.97
Standard error	2.91
Standard deviation	10.09
Minimum	4.55
Maximum	38.76
Skewness	1.75
Standardized skewness	2.48
Kurtosis	3.18
Standardized kurtosis	2.25

patients is normally distributed and is shown in Fig.5.4.14.

The proportion of large bowel cancer patients with high P450IA activity (ratio >26) is shown in Table 5.4.10.

8.3% of the patients with colorectal carcinoma had high P450IA activity (Fig.5.4.13). This was not significantly different to the proportion of control subjects with high P450IA activity (9%) ($p>0.05$). All of the patients with large bowel cancer were nonsmokers. The proportion of these patients with high P450IA activity was also not significantly different from the proportion of control nonsmokers (6.5%) with high P450IA activity ($p >0.05$).

Table 5.4.10 - The proportion of colorectal cancer patients with high P450IA activity

Group	P450IA activity			
	High	%	Low	%
Colorectal cancer	1/12	(8.3%)	11/12	(91.7%)
Control	25/277	(9.0%)	252/277	(91.0%)
-Nonsmokers	12/185	(6.5%)	173/185	(93.5%)

P450IA2 Activity

The frequency distribution histogram for P450IA2 activity in 35 patients diagnosed with FAP is depicted in Fig.5.4.15. The summary statistics are shown in Table 5.4.11.

Table 5.4.11 - Summary Statistics for P450IA2 activity in 35 patients with FAP

Median	108.21
Mean	190.02
Standard error	35.85
Standard deviation	212.08
Minimum	6.25
Maximum	846.67
Skewness	1.99
Standardized skewness	4.80
Kurtosis	3.32
Standardized kurtosis	4.01

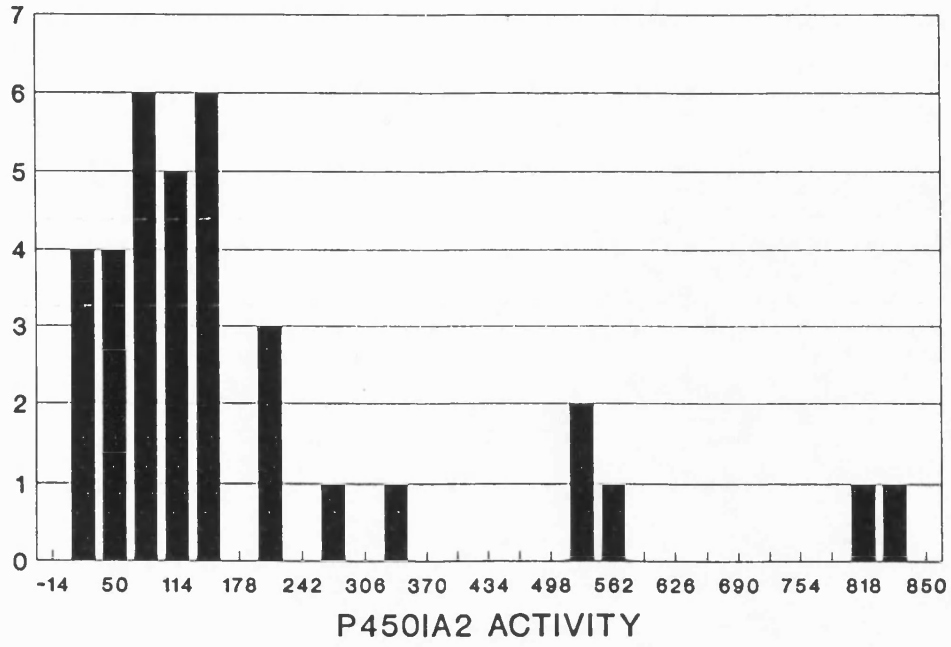


Figure 5.4.15 - Frequency distribution histogram of P450IA2 activity in 35 patients with FAP.

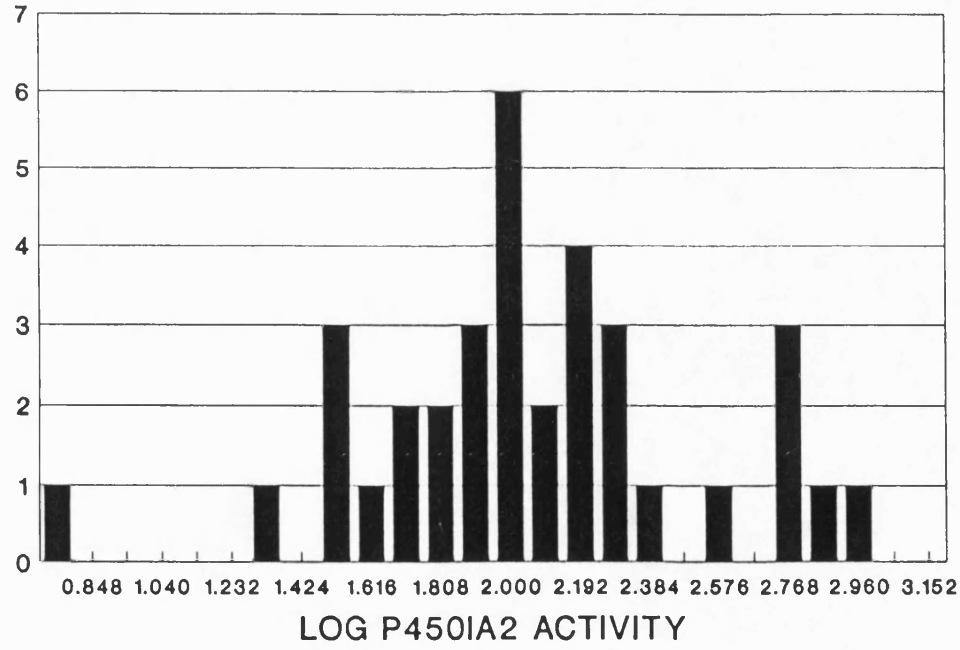


Figure 5.4.16 - Log frequency distribution histogram of P450IA2 activity in 35 patients with FAP.

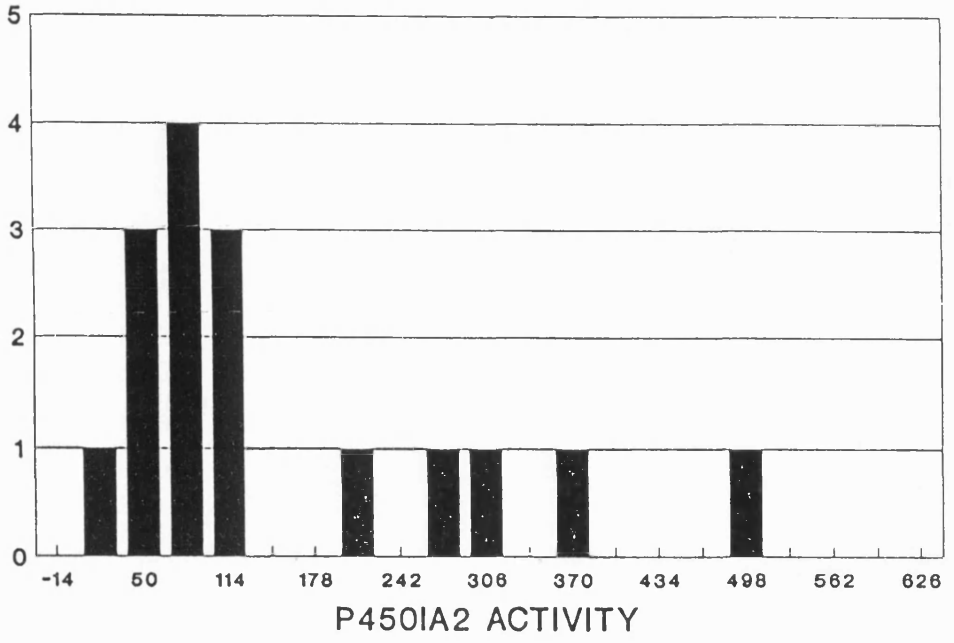


Figure 5.4.17 - Frequency distribution histogram of P450IA2 activity in 16 relatives at risk for FAP.

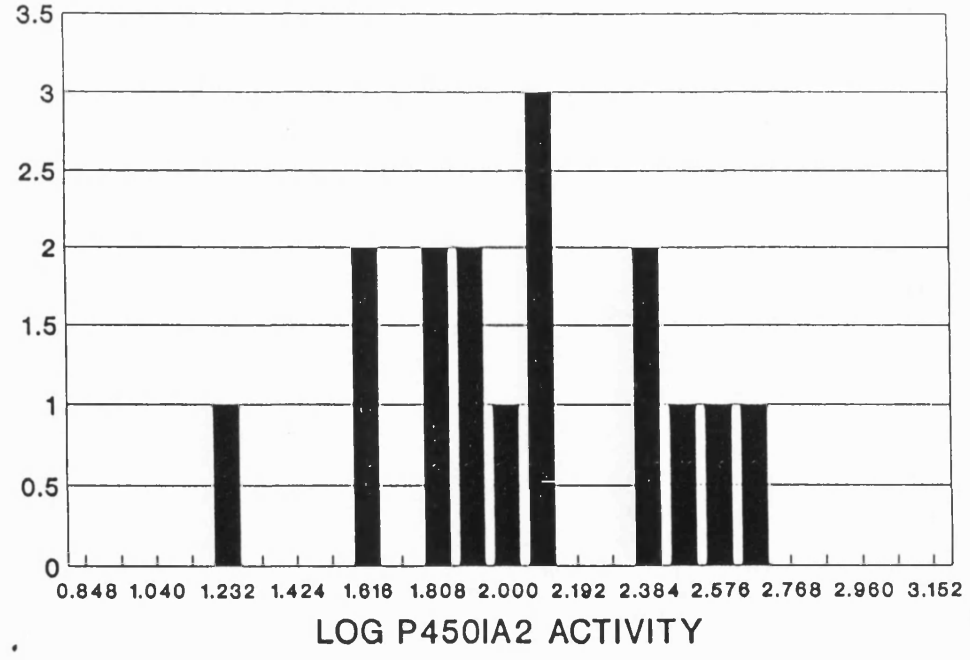


Figure 5.4.18 - Log frequency distribution histogram of P450IA2 activity in 16 relatives at risk for FAP.

135-fold variation in P450IA2 activity existed. It can be seen from the histogram and Table 5.4.11 that the distribution shows very large degrees of positive skewness, deviates from normailty and is probably bimodal (Chi-square test, $p<0.00001$; K-S test, $p<0.01$; Lilly-Fors test, $p<0.01$). However, the logged data were found to be unimodally distributed (Fig.5.4.16).

The frequency distribution histogram for P450IA2 activity in 16 relatives at risk for FAP is depicted in Fig.5.4.17. The summary statistics for this distribution are shown in Table 5.4.12. 28-fold variation in enzyme activity occurs in only 16 subjects. It can be seen from the histogram and Table 5.4.12 that the distribution is positively skewed and possibly bimodal (Lilly-Fors test, $p<0.01$). The log frequency distribution depicted in Fig.5.4.18 was found to be unimodally distributed.

Table 5.4.12 - Summary Statistics for P450IA2 activity in 16 relatives at risk for FAP

Median	104.02
Mean	157.04
Standard error	34.58
Standard deviation	138.31
Minimum	18.04
Maximum	502.45
Skewness	1.38
Standardized skewness	2.25
Kurtosis	1.25
Standardized kurtosis	1.02

The proportion of patients with FAP and their relatives at risk for the disease with high P450IA2 activity is shown in Table 5.4.13.

Table 5.4.13 - The proportion of FAP patients and their relatives at risk for FAP with high P450IA2 activity

Group	P450IA2 activity			
	High	%	Low	%
FAP	10/35	(28.6%)	25/35	(71.4%)
-Smokers	4/ 5	(80.0%)	1/ 5	(20.0%)
-Nonsmokers	5/25	(20.0%)	20/25	(80.0%)
At Risk	5/16	(31.3%)	11/16	(68.7%)
-Smokers	3/ 6	(50.0%)	3/ 6	(50.0%)
-Nonsmokers	2/10	(20.0%)	8/10	(80.0%)
Control	42/254	(16.5%)	212/254	(83.5%)
-Smokers	19/ 70	(27.1%)	51/ 70	(72.9%)
-Nonsmokers	23/184	(12.5%)	161/184	(87.5%)

28.6% of the patients with FAP had high P450IA2 activity (Fig.5.4.15). This was not significantly different from the proportion of control subjects with high P450IA2 activity (16.5%) ($p>0.05$). The number of smoking FAP patients with high P450IA2 activity was greater than the number of nonsmoking patients ($p<0.01$). The number of smoking FAP patients with high P450IA2 activity was greater than the number of control smokers

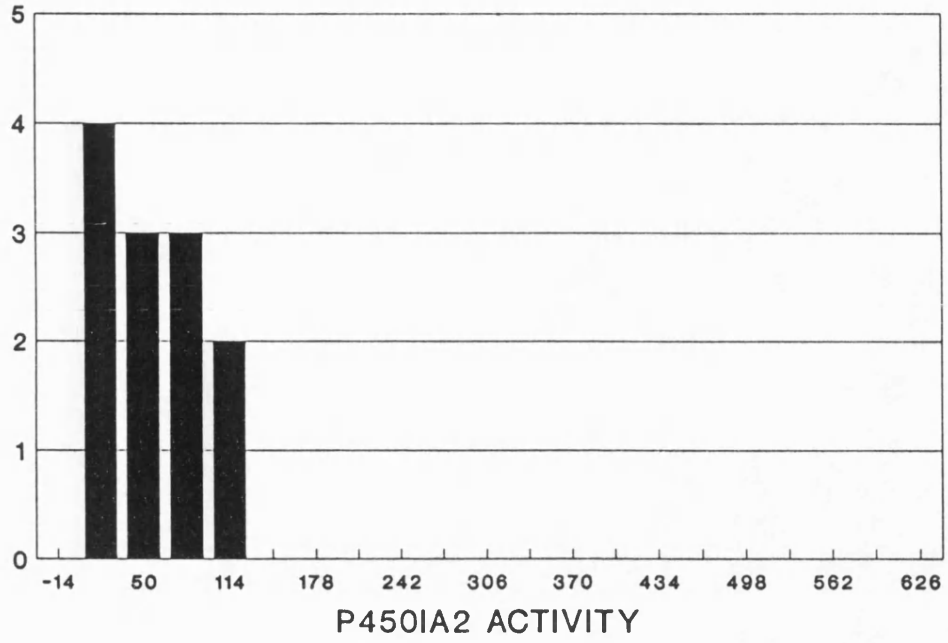


Figure 5.4.19 - Frequency distribution histogram of P450IA2 activity in 12 patients with colorectal cancer.

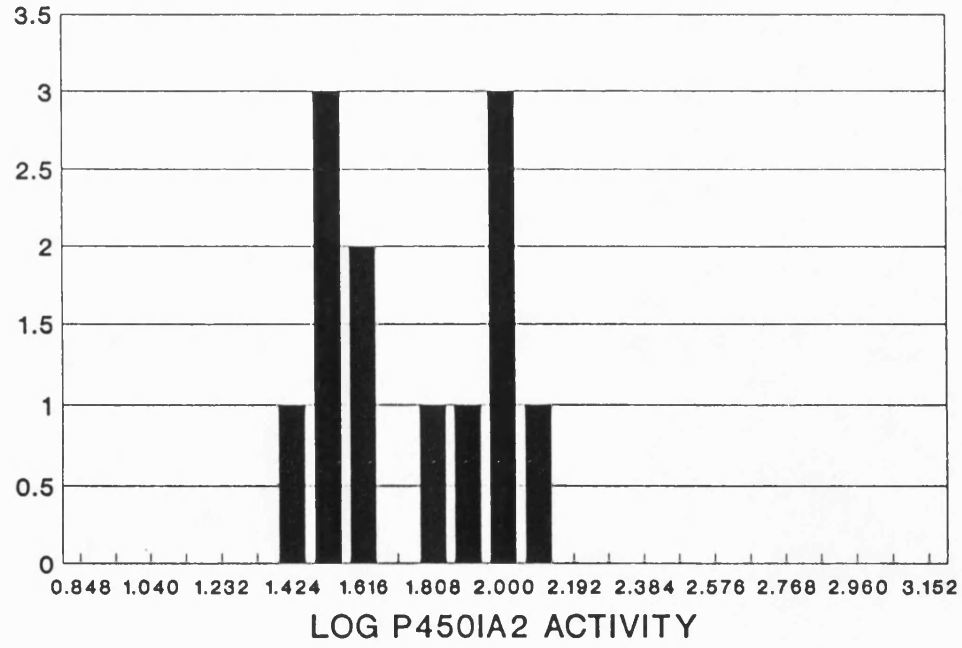


Figure 5.4.20 - Log frequency distribution histogram of P450IA2 activity in 12 patients with colorectal cancer.

($p<0.05$). However, the proportion of nonsmoking FAP patients with high P450IA2 activity was not significantly different from that in nonsmoking control subjects ($p>0.05$). The number of FAP patients with high P450IA2 activity was not significantly different from that in the relatives at risk for FAP ($p>0.05$).
The proportion of relatives at risk for FAP with high P450IA2 activity was 31.3% (Fig.5.4.17) which was not significantly different from the proportion of control subjects with high P450IA2 activity ($p>0.05$). The number of smoking relatives at risk, with high P450IA2 activity was not significantly different from the number of nonsmoking relatives ($p>0.05$). Neither the smoking or nonsmoking groups of relatives at risk had a greater proportion of subjects with high P450IA2 activity than the control smokers or nonsmokers ($p>0.05$).

The frequency distribution histogram for P450IA2 activity in 12 patients diagnosed with colorectal cancer is depicted in Fig.5.4.19. The summary statistics for this distribution are shown in Table 5.4.14. 5-fold variation in P450IA2 activity existed. It can be seen from the histogram and Table 5.4.14 that the distribution is only very slightly skewed results and seems to fit a normal distribution. The log frequency distribution for P450IA activity in the 12 colorectal cancer patients is normally distributed and is shown in Fig.5.4.20.
The proportion of large bowel cancer patients with high P450IA2 activity (ratio >162) is shown in Table 5.4.15. Although none of the patients with large bowel cancer had high P450IA2 activity (Fig.5.4.19), this was not significantly different from that in the control population (16.5%) ($p>0.05$). Since all of the

Table 5.4.14 - Summary Statistics for P450IA2 activity in 12 patients with colorectal cancer

Median	51.88
Mean	64.76
Standard error	10.60
Standard deviation	36.72
Minimum	23.89
Maximum	129.94
Skewness	0.53
Standardized skewness	0.74
Kurtosis	-1.26
Standardized kurtosis	-0.89

Table 5.4.15 - The proportion of colorectal cancer patients with high P450IA2 activity

Group	P450IA2 activity			
	High	%	Low	%
Colorectal cancer	0/ 12	(0%)	12/ 12	(100%)
Control	42/254	(16.5%)	212/254	(83.5%)
-Nonsmokers	23/184	(12.5%)	161/184	(87.5%)

colorectal cancer patients were nonsmokers, they were compared to the nonsmoking control subjects. There was no significant difference between the proportion of subjects with high P450IA2

activity between these two groups ($p>0.05$).

5.4.5 Discussion

P450IA2 activity in 254 control volunteers was shown to vary 77-fold in the present study (Section 5.1). Such large variation in the metabolism of aminobiphenyl (Butler et al, 1989a), 2-naphthylamine (Hammons et al, 1985), phenacetin (Shimada et al, 1989; Sesardic et al, 1988) caffeine (Butler et al, 1989b) and in the activity of P450IA2 itself are frequently described (Wrighton et al, 1986; Davies et al, 1987; Sesardic et al, 1988; Ikeya et al, 1989). Many of these workers have suggested that P450IA2 may be subject to genetic polymorphism, in order to explain such large interindividual variation. Since the N-oxidation of arylamines is a critical step in the metabolic activation of these carcinogens, the existence of a genetic polymorphism in P450IA2 activity would suggest that some individuals with high activity may have a genetic predisposition to arylamine-induced cancers.

Patients with FAP were found to have higher P450IA2 activity than healthy controls, even in the presence of large variation in P450IA2 activity observed in healthy subjects. Not all FAP patients had high P450IA2 activity, suggesting that this enzyme system is not solely responsible for any abnormal hepatic metabolism of carcinogens in FAP. This is not surprising since CYPIA2 is inherited on chromosome 15, whereas FAP is an autosomal dominant condition involving chromosome 5. This difference in the pattern of inheritance does not, however, preclude a relationship between FAP and P450IA2 activity. It has been shown in the past that inherited disorders, such as FAP can

be influenced by environmental factors. For example, American patients with multiple polyposis have far fewer gastric polyps than Japanese patients. It is thought that the Japanese diet, already incriminated in the high rate of stomach cancer, may enhance the expression of gastric neoplasm by the polyposis gene (Utsunomiya et al, 1974).

The response of FAP patients to the inductive effects of cigarette smoke was similar to that observed in control subjects, in that smoking patients with FAP had a greater P450IA2 activity than nonsmokers and the proportion of high P450IA2 activity in smokers was greater than in nonsmokers. In fact, the 5 smoking patients with FAP had a higher P450IA2 activity than 70 control smokers, which might suggest that factors in addition to cigarette smoke are involved in the increase in P450IA2 activity observed in FAP patients. The 25 nonsmoking patients with FAP had higher P450IA2 activities than control nonsmokers, to such an extent that they even tended to have higher mean activities than control smokers. Undoubtedly, the most remarkable finding in this study was the fact that P450IA2 activity varied 135-fold in the FAP patients studied, in contrast to the 77-fold variation observed in control volunteers. Taken together these results suggest that genetic factors may play a more important role than environmental factors, in determining the degree of P450IA2 activity of some individuals. Moreover, it appears that genetic differences in P450IA2 activity, in addition to a genetic defect involving chromosome 5, may be involved in FAP susceptibility.

Hepatic P450IA2 is responsible for the N-oxidation and thus metabolic activation of aromatic amines (Thorgeirsson & Nebert,

1977; Kamataki et al, 1983; Hammons et al, 1985; Shimada & Okuda, 1988; Shimada et al, 1989; Guengerich et al, 1990), promutagens and procarcinogens (Aoyama et al, 1989; Gonzalez et al, 1990). The enzyme itself is induced by such compounds (Degawa et al, 1989) and it appears that a relationship exists between the carcinogenicity of aromatic amines and their ability to induce hepatic P450IA2 in the rat (Ayrton et al, 1990a, b). Ring-hydroxylation and conjugation of arylamines are detoxification pathways.

Patients with bladder cancer more commonly have high P450IA2 activity (Bartsch et al, 1990). N-OH-arylamines formed by hepatic P450IA2 are transported in the form of glucuronides (Poupko et al, 1979) in the blood stream to the kidney, where they are filtered and subsequently reach the urinary bladder lumen. At slightly acidic pH, the conjugates are hydrolysed (Flammang et al, 1985) and the N-OH-arylamines, which are much more potent carcinogens than the parent compounds (Miller & Miller, 1981) are capable of reacting directly with DNA in the urothelium. For colorectal carcinogenesis by arylamines, metabolic N-oxidation by hepatic P450IA2 is likewise regarded as the important activation step (Kadlubar & Hammons, 1987). Conjugation by hepatic N-glucuronidation is thought to result in biliary transport of the metabolites to the colonic lumen (Poupko et al, 1979; Meerman et al, 1982), where bacterial beta-glucuronidases can regenerate N-OH-arylamines (Kadlubar & Beland, 1985). These findings suggest that an analogous situation might occur in FAP.

FAP is a premalignant condition conferring a high risk of duodenal cancers and adenomas. Such neoplasms cluster around the

ampulla of Vater (Spigelman et al, 1991) but are found rarely in the stomach. When gastric adenomas are present, they usually occur in the distal stomach, in association with duodeno-gastric reflux of bile. This pattern of foregut tumours in patients with FAP could be explained by the transport of carcinogens in bile. One may hypothesise that patients with FAP have greater concentrations of carcinogenic compounds excreted into bile, due to their higher hepatic P450IA2 activity than control subjects. This theory is supported by the animal model for colorectal cancer. A similar distribution of intestinal tumours occurs in the animal model, when a carcinogen, adminstered subcutaneously or orally, is excreted into the bile duct after undergoing hepatic metabolism.

As P450IA2 is not induced extrahepatically it is probably not present in human bladder (Domin et al, 1984) or gut (Vang et al, 1990). Rather, it appears that the hepatic activity of P450IA2 is important in the onset of bladder cancer and possibly FAP. The fact that P450IA2 activity was unaltered in patients with carcinoma of the lung, head and neck and leukaemia suggests that high P450IA2 activity in the FAP patients is not an effect of malignancy per se.

The 16 relatives of FAP patients did not have polyps at the time of the caffeine test but were considered to be at risk for FAP because it was not known whether they carried the mutant gene for FAP development. While this small group of relatives also had higher P450IA2 activities than control subjects, the proportion of them with high P450IA2 activities was not different to that in controls. Also, there was no significant

difference in P450IA2 activity between the smoking and nonsmoking relatives. While the smoking relatives tended to have higher P450IA2 activities than control smokers, which might suggest that factors other than cigarette smoke can affect P450IA2 in this group of subjects as well as FAP, the nonsmoking relatives had similar P450IA2 activities to nonsmoking control subjects. The disparity of results between FAP patients and their relatives at risk for FAP is unlikely to be due to the younger mean age of the group at risk for FAP because P450IA2 activity is unaltered by subject age. The difference is likely due to the fact that not all of the relatives tested would have had the mutant gene for FAP development. The 50% risk of inheriting the gene means that while some of the relatives will inevitably develop FAP, and therefore probably have high P450IA2 activity, others will not have inherited the gene and would therefore be expected to have normal levels of P450IA2 activity. As overall, P450IA2 activity is greater in the control group of relatives at risk for FAP than control subjects, one might hypothesise that more relatives have the mutant gene than have not. It is tempting to speculate that the relatives with high P450IA2 activity will eventually develop FAP.

P450IA activity was not altered in FAP or relatives at risk for FAP compared to control subjects. Smoking FAP patients tended to have higher P450IA activity than nonsmokers, as expected following the observation in control subjects that both P450IA and IA2 are induced by cigarette smoke. A similar pattern was observed between smoking and nonsmoking relatives at risk for FAP. The fact that P450IA2 activity was grossly altered in FAP whereas P450IA was not significantly different from that in

control volunteers again suggests that P450IA and IA2 are different isozymes. While P450IA activity appears to important in the susceptibility of an individual to carcinomas of the lung, head and neck, P450IA2 activity does not (Chapter 4.2). Conversely, while P450IA2 activity appears to be involved in bladder cancer and possibly FAP, we have shown that P450IA activity is not altered in this latter group of patients. Other workers have found that P450IA1 activity, unlike P450IA2 activity, is not involved in cancer of the renal pelvis and ureter (Trell et al, 1977) or urinary bladder cancer (Kellermann et al, 1973b; Trell et al, 1978; Paigen et al, 1979).

In contrast to FAP patients and their relatives at risk for FAP, P450IA2 and P450IA activities in patients with large bowel cancer were not different from that in the control population. Weston et al (1991) found elevated amounts of arylamine-haemoglobin adducts in nonsmoking patients with colon cancer, compared to controls which suggests that arylamines are involved in colorectal cancer. Results from the present study however suggest that high P450IA2 is not entirely responsible for intestinal cancer risk; other polymorphic enzyme systems such as N-acetyltransferase are also involved in the metabolism of carcinogenic arylamines and may play an important role in colorectal cancer.

While the patients with cancer of the large bowel have a much higher mean age than the control population, the lack of high P450IA2 activity in this group of patients is probably not an age-related effect, as we have shown that P450IA2 activity and subject age are unrelated. Nonetheless, cancer of the colon and

rectum increases with age, probably as a result of a constantly present carcinogen in the environment (Moller-Jensen, 1983). Although first degree relatives of patients with colorectal cancer have an increased risk for colonic cancer, and familial clusters of colorectal cancer cases have frequently been observed (Burt et al, 1985), it is known that environmental factors, such as the diet play an important role in bowel cancer. Mutagens such as heterocyclic arylamines, which are present in charred surfaces of fish and beef, grilled chicken and roasted coffee beans (Kawachi et al, 1979; Sugimura, 1986) are activated by P450IA2 (Battula et al, 1990) and are capable of producing colon tumours in animals (Sugimura, 1986). Meats prepared by smoking, curing and barbecueing have also been found to be positively associated with colon cancer (Worlleb et al, 1990). In addition, it has been hypothesised that the amount of dietary fat determines the composition of the gut microflora, which can activate carcinogens in the large bowel (Reddy et al, 1980). Fecal bacterial 7-alpha-dehydroxylase can convert cholic acid to secondary bile acids which may act as colon tumor promoters and beta-glucuronidase can hydrolyse N-OH-arylamines conjugates in the bowel. Both enzymes are present in high concentrations in high fat/ meat diets. At present, it remains unknown whether the carcinogen exists as such in the diet characteristic of populations at high risk for bowel cancer, or whether the active compounds are formed by hepatic enzymes and gut flora from metabolites of the affluent diet.

CHAPTER SIX

N-ACETYLTRANSFERASE ACTIVITY IN HUMAN VOLUNTEERS

6.1 N-ACETYLTRANSFERASE ACTIVITY IN HEALTHY INDIVIDUALS

6.1.1 Introduction

N-acetyltransferase (NAT) is subject to genetic polymorphism (Evans et al, 1960) such that some subjects are slow acetylators while others are fast acetylators (Weber & Hein, 1985). Most tests for acetylator phenotyping involve administration of a drug, such as sulphamethazine and monitoring its metabolism via plasma and urine sampling (Evans, 1989). It may be possible to use a simpler, noninvasive test to determine acetylator phenotype in healthy populations, by measuring a caffeine metabolite ratio (Tang et al, 1991). By giving caffeine as a probe drug to healthy volunteers and studying the urinary excretion of its metabolites, the effects of both environmental and genetic factors on N-acetylation capacity were examined.

6.1.2 Results

Following the observation that the caffeine metabolite ratio used to determine NAT activity was not altered by the amount of caffeine consumption (Section 4.1) or the time of urine collection (Section 4.2), results for NAT activity in 0-8h and 2-6 h tests were combined. The mean NAT activity determined in 277 control volunteers was 0.45 $\pm$ 0.01 and ranged between 0.077 and 0.886 - an eleven-fold range in activity. Of the 277 subjects studied, 59% were classified as slow acetylators and 41% as rapid acetylators, the cut-off point in enzyme activity being 0.48.

6.1.3 Effect of Age

277 healthy subjects with recorded dates of birth were studied. Their mean age was 40.42 ± 0.96 y and ranged between 17 and 91 y. No correlation between subject age and the degree of NAT activity was found ($r=-0.0994$, $p>0.05$). The effect of age was also studied for each phenotype separately (Evans, 1989). The mean age of the 160 slow acetylators was 42.54 ± 1.40 y and ranged between 18 and 90 y. The mean age of the 117 rapid acetylators was 37.50 ± 1.18 y and ranged between 17 and 62 y. While age was found to have no effect on the degree of NAT activity in subjects with the rapid acetylator phenotype ($r=-0.043$, $p>0.5$), the degree of NAT activity in the slow phenotype subjects appeared to decrease with age ($r=-0.1334$, $p=0.09$). NAT activity in elderly subjects (>60 y) was highly significantly less than that determined in subjects under 60 y of age ($p<0.0001$).

In addition, the effect of age on NAT activity was studied separately for elderly and young subjects. For the 29 elderly subjects (mean age 72.90 ± 1.70 y; range 60 - 90 y) no significant correlation between age and NAT activity existed ($r=-0.0538$, $p>0.5$). Similarly, age was not found to effect NAT activity when the younger group of subjects aged <60 y were studied (mean age 36.62 ± 0.75 y; range 17 - 59 y) ($r=0.0207$, $p>0.5$).

6.1.4 Effect of Body Mass Index

254 subjects with recorded heights (m) and weights (kg) were studied. Body mass index values for 129 females ranged between 17.40 and 52.14 (23.72 ± 0.37). The values for 125 males ranged

between 17.04 and 34.33 (24.45 ±0.26) and were not significantly different from the values determined in females ($p>0.1$). No correlation was found between BMI values and the activity of NAT ($r=-0.0874$, $p>0.1$).

BMI values for the elderly subjects (mean 24.52 ± 0.57; range 17.91 - 31.16) were not significantly different from values determined in subjects under 60 y of age (mean 24.01 ± 0.24; range 17.04 - 52.14) ($p>0.1$). No correlation between NAT activity and BMI values was found in 29 elderly subjects ($r=-0.1049$, $p>0.5$) or in 248 subjects under 60 y of age ($r=-0.0976$, $p>0.1$).

6.1.5 Effect of Subject Gender

The effect of subject gender was examined in 129 males and 119 females who were under the age of 60 y, as differences in liver mass and hormonal status between the sexes may alter enzyme activities. Of the females, 42 were using oral contraceptive steroids (OCS) or hormone replacement therapy (HRT), while 77 were non-OCS users. The results are shown in Table 6.1.1.

NAT activity was not significantly different between males and females, whether males were compared to non-OCS user females ($p>0.5$) or the whole female population, including OCS and non-OCS users ($p>0.1$) (Fig.6.1.1).

In addition, no significant difference in NAT activity was found between 16 males and 13 females aged 60 y or more ($p>0.5$) (Table 6.1.2). None of the females were taking either OCS or HRT.

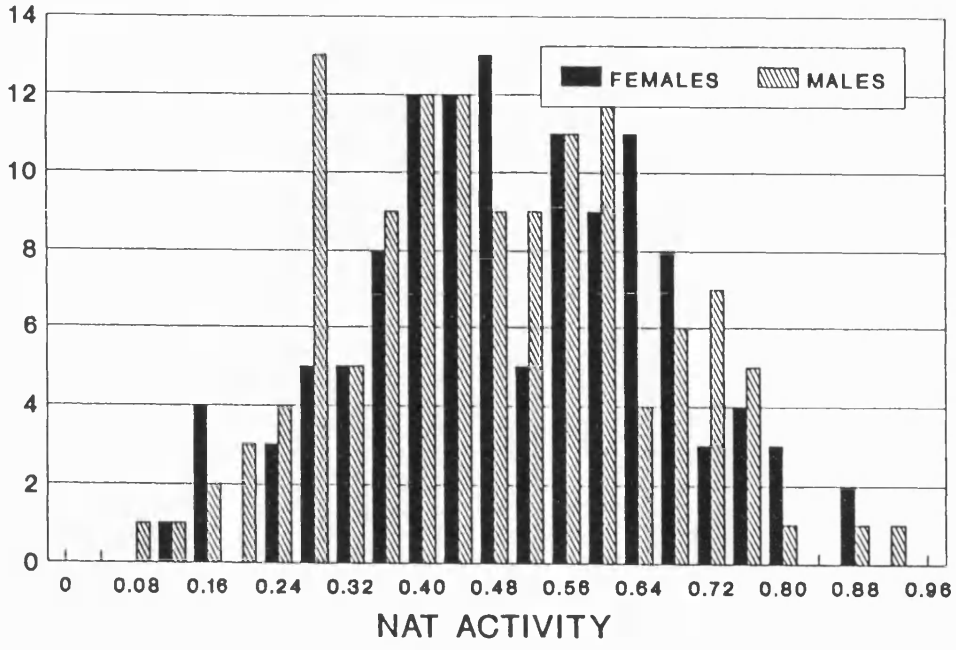


Figure 6.1.1 - Effect of subject gender on NAT activity.

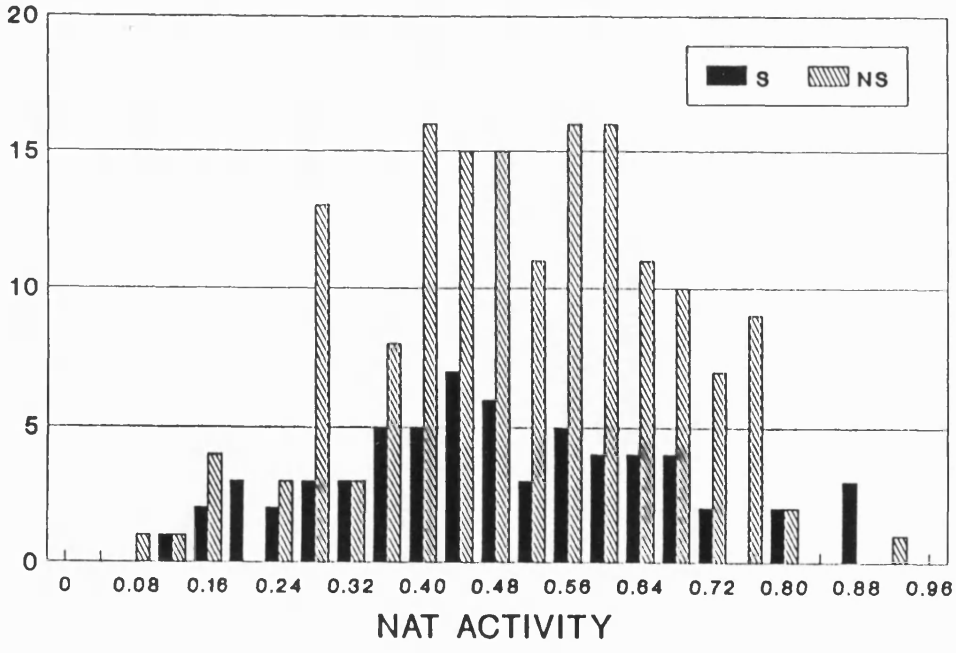


Figure 6.1.2 - Effect of cigarette smoke on NAT activity.

Table 6.1.1 - NAT activity in male and female populations under 60 y of age

Group	n	Mean activity ± SEM	Median	Range
Males	129	0.459 ± 0.015	0.446	0.077 -0.886
Females				
(All)	119	0.482 ± 0.015	0.468	0.104 -0.864
(Non-OCS)	77	0.463 ± 0.018	0.459	0.104 -0.864

Table 6.1.2 - NAT activity in male and female populations aged 60 y or more

Group	n	Mean activity ± SEM	Median	Range
Males	16	0.329 ± 0.037	0.296	0.121 - 0.667
Females	13	0.289 ± 0.026	0.265	0.162 - 0.475

6.1.6 Effect of Oral Contraceptive Steroids

In order to determine any effect of OCS/HRT use on NAT activity, 42 females using OCS or HRT were compared to 77 non-OCS users (Table 6.1.3). Mean NAT activities were not significantly different between the two groups ($p>0.05$).

Table 6.1.3 - NAT activity in OCS and non-OCS using females under 60 y of age

Group	n	Mean activity ± SEM	Median	Range
OCS	42	0.517 ± 0.026	0.528	0.140 - 0.851
Non-OCS	77	0.463 ± 0.018	0.459	0.104 - 0.864

Table 6.1.4 - NAT activity in smoking and nonsmoking healthy populations under 60 y of age

Group		n	Mean activity ± SEM	Median	Range
Smokers		64	0.460 ±0.023	0.443	0.104 -0.866
Male		31	0.423 ±0.031	0.410	0.142 -0.844
Female	-OCS	18	0.524 ±0.039	0.580	0.213 -0.851
	-nonOCS	15	0.460 ±0.056	0.458	0.104 -0.864
	-all	33	0.495 ±0.033	0.525	0.104 -0.864
Nonsmokers		162	0.486 ±0.013	0.494	0.077 -0.886
Male		79	0.493 ±0.019	0.516	0.077 -0.886
Female	-OCS	24	0.512 ±0.034	0.491	0.140 -0.799
	-nonOCS	62	0.464 ±0.019	0.461	0.148 -0.781
	-all	86	0.478 ±0.017	0.466	0.140 -0.799

6.1.7 Effect of Cigarette Smoke

As cigarette smoke is an inducer of certain P450 enzymes, its effect on NAT activity was also studied in subjects under 60 y of age. The results are shown in Table 6.1.4. NAT activity in smoking subjects was not significantly different from that determined in healthy nonsmokers ($p>0.1$) (Fig.6.1.2). While male smokers tended to have lower NAT activity than male nonsmokers ($p<0.05$) NAT activity in female smokers was not significantly different from female nonsmokers, whether the women were using OCS ($p>0.5$) or not ($p>0.5$). In addition, NAT activity in female smokers was similar whether the women were using OCS or not ($p>0.1$). NAT activity in female nonsmokers was also unaltered by OCS administration ($p>0.1$).

Table 6.1.5 - NAT activity in smoking and nonsmoking healthy populations aged 60 y or more

Group	n	Median	Mean activity ± SEM	Range
Smokers	6	0.292	0.320 ± 0.076	0.121 - 0.667
Male	4	0.292	0.343 ± 0.116	0.121 - 0.667
Female	2	0.274	0.274 ± 0.050	0.224 - 0.323
Nonsmokers	23	0.292	0.309 ± 0.023	0.145 - 0.592
Male	12	0.296	0.325 ± 0.035	0.145 - 0.592
Female	11	0.265	0.292 ± 0.030	0.162 - 0.475

NAT activity was also determined in smokers and nonsmokers, aged

60 y or more (Table 6.1.5). NAT activity in healthy elderly smokers was not significantly different from that measured in healthy elderly nonsmokers ($p > 0.5$). This was also the case when elderly male ($p > 0.5$) and female smokers and nonsmokers ($p > 0.5$) were studied separately. NAT activity in male smokers was not significantly different from that in female smokers ($p > 0.5$). Similarly, NAT activity in male nonsmokers was not significantly different from that in female nonsmokers ($p > 0.5$).

6.1.8 Genetic Control of N-Acetyltransferase Activity

The frequency distribution for NAT activity in 277 healthy volunteers is depicted in Fig.6.1.3. The summary statistics for this distribution are shown in Table 6.1.6.

Table 6.1.6 - Summary Statistics for NAT activity in a population of 277 healthy subjects

Median	0.443
Mean	0.450
Standard error	0.010
Standard deviation	0.170
Minimum	0.077
Maximum	0.886
Skewness	0.130
Standardized skewness	0.884
Kurtosis	-0.578
Standardized kurtosis	-1.964

The mean NAT activity is 0.45 ± 0.01 and ranged from 0.077 to 0.886 - an 11-fold range in activity. It can be seen from the histogram and Table 6.1.6 that the distribution is only slightly positively skewed. The following are worth noting:

a) The standardized coefficient indicates that this degree of skewness is probably not different from that expected from a normal distribution. The distribution for NAT activity does show a degree of kurtosis however, and the negative standardized kurtosis coefficient suggests that the distribution is flatter, with shorter tails than would be expected from a normal distribution.

b) Although there are no clear breaks in the normal probability plot (Fig.6.1.4), an inflection in the curve exists at a point equivalent to an NAT activity of 0.48. It can also be seen from the frequency histogram that different modes are present within the normal distribution range, such that a ratio value of 0.48 appears to separate the two major modes observed (Fig.6.1.3).

c) The Chi-square and K-S tests ($p>0.1$) however, indicate that NAT activity in a population of 277 control volunteers is likely to fit a normal distribution.

d) The Lilly-Fors test also suggests that the data are unlikely to fit a bimodal curve ($p>0.05$).

These results were unexpected following the observation by several other workers that NAT activity is subject to genetic polymorphism and bimodally distributed.

Frequency histograms of NAT activity were also studied separately for 248 subjects aged under 60 y of age and 29 elderly volunteers. There was a clearer separation of the major

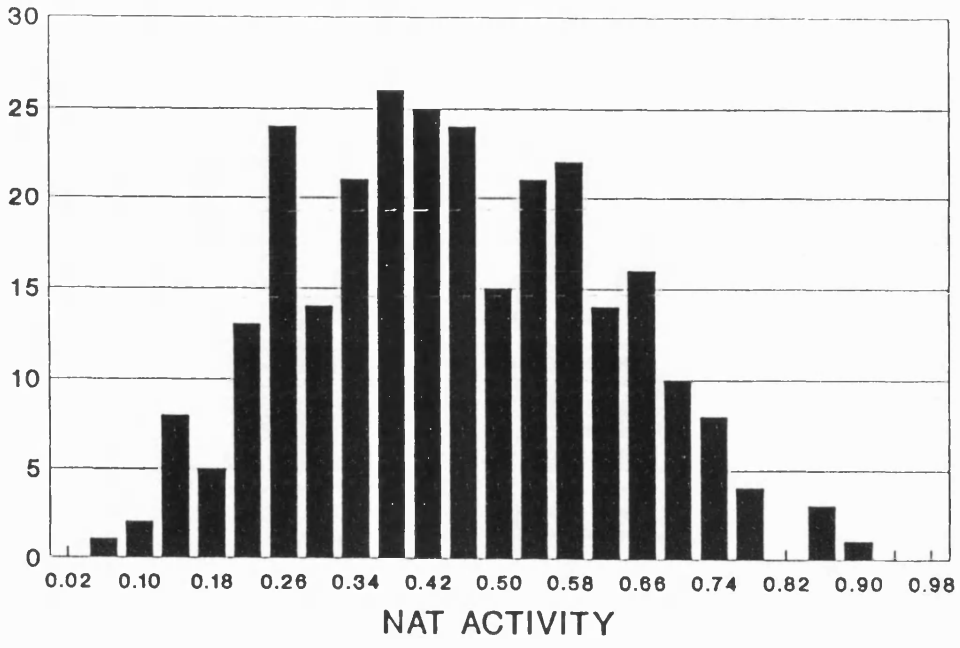


Figure 6.1.3 - Frequency distribution histogram of NAT activity in 277 controls.

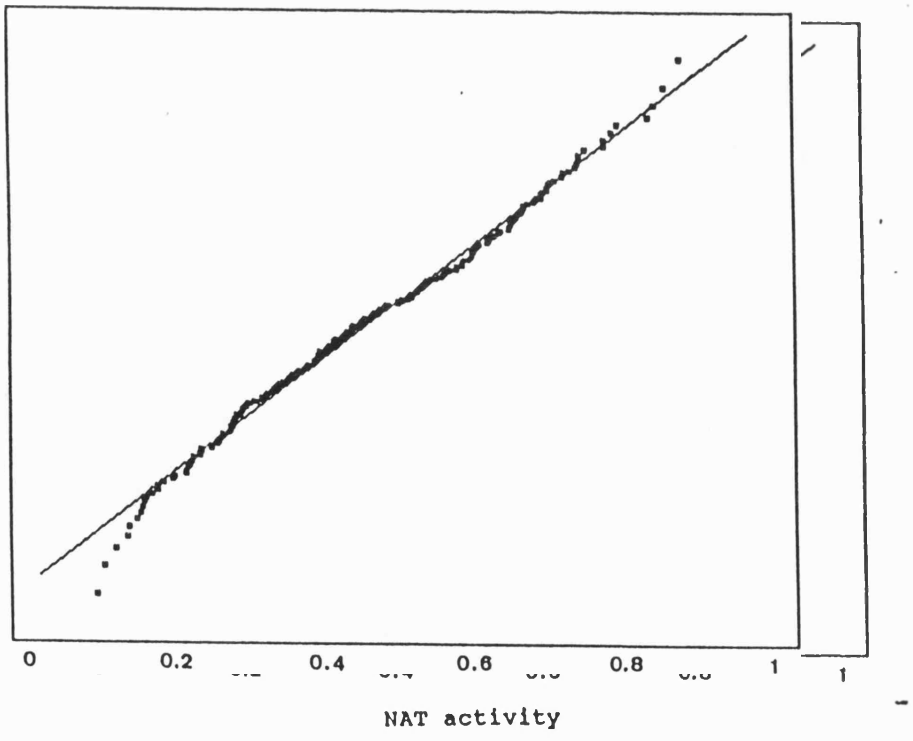


Figure 6.1.4 - Normal probability plot of NAT activity in 277 controls.

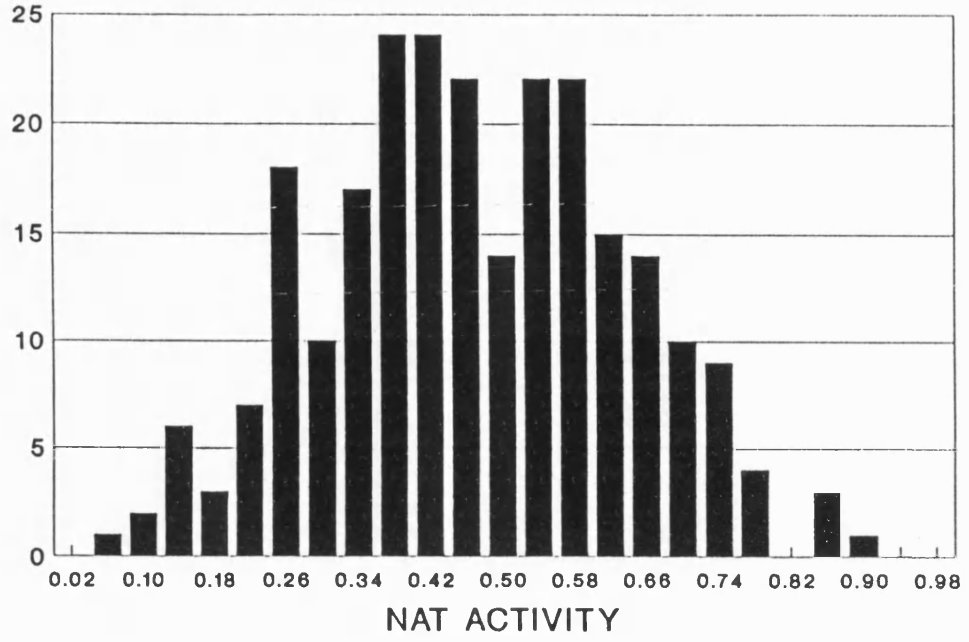


Figure 6.1.5 - Frequency distribution histogram of NAT activity in 248 subjects, under 60 y of age.

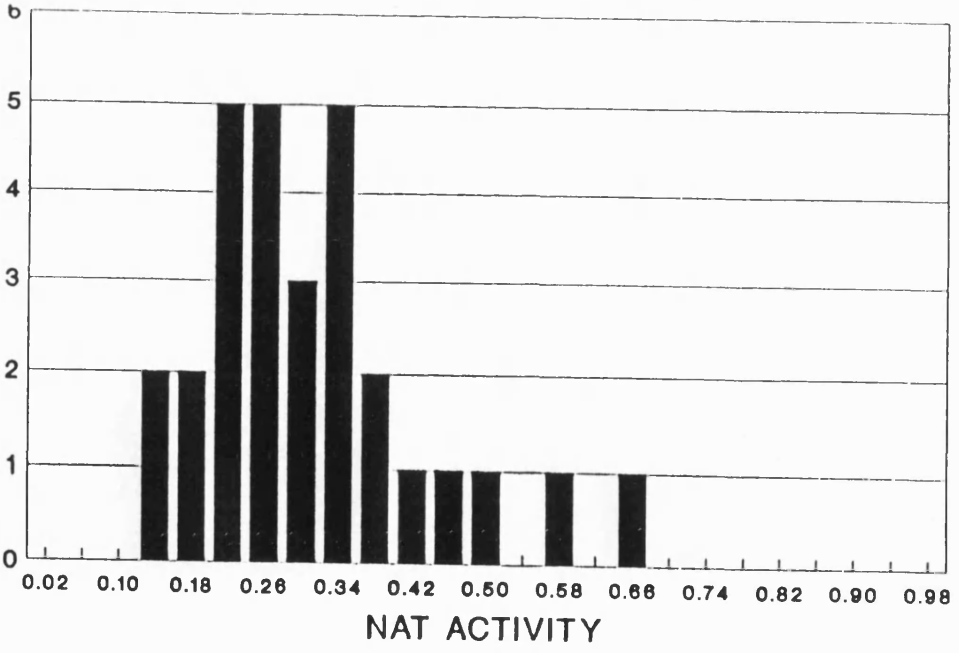


Figure 6.1.6 - Frequency distribution histogram of NAT activity in 29 subjects, over 60 y of age.

modes of NAT activity in the younger subjects (Fig.6.1.5) than when the whole population was studied. However, statistical tests performed on the data and the Lilly-Fors test ($p>0.05$) indicated that NAT activity is unlikely to be bimodal. When the frequency histogram (Fig.6.1.6) of NAT activity were studied for 29 elderly subjects, bimodality of NAT activity was not observed.

In order to detect the different phenotypes which are known to exist for NAT activity, the data were logged, as bimodality of distributions is often easier to observe when log transformation of the data is performed (Jackson et al, 1986). The frequency distribution for log NAT activity and the summary statistics for this distribution in 277 healthy subjects are shown in Fig.6.1.7 and Table 6.1.8, respectively.

Table 6.1.8 - Summary Statistics for log NAT activity in a population of 277 healthy subjects

Mean	-0.380
Standard error	0.011
Standard deviation	0.190
Minimum	-1.116
Maximum	-0.053
Skewness	-0.976
Standardized skewness	-6.628
Kurtosis	1.147
Standardized kurtosis	3.896

This distribution is highly negatively skewed and shows a large degree of kurtosis. Both standardized coefficients and statistical tests indicate that the data deviates from normality and may be bimodal in nature (Chi-square test, $p<0.0001$; K-S test, $p=0.09$; Lilly-Fors test $p<0.01$). Although two distinct modes are not clearly evident in the log frequency curve for NAT activity in 277 healthy subjects, there appears to be an antimode at a metabolite ratio of -0.30 (Fig.6.1.7). This value equates to an NAT activity of 0.50 when antilogged, which fits well with the point of inflection of 0.48 evident from the normal probability plot (Fig.6.1.4).

Due to the effect of age on NAT activity, log frequency distributions for subjects aged under 60 y were studied separately from subjects >60 y. For the younger subjects there was a clearer separation of the modes in the log frequency histogram at a metabolite ratio value equivalent to 0.501 (Fig.6.1.8). The chi-square test ($p<0.00001$), K-S test ($p<0.05$) and Lilly-Fors test results ($p<0.01$) indicated that NAT activity for these younger subjects is significantly different from normal and probably bimodal in nature. The log frequency distribution for NAT activity in 29 elderly subjects is shown in Figs.6.1.9. The antimode of -0.36 is lower in the elderly than that observed for the younger subjects and equates to a metabolite ratio of 0.44. Therefore, for subjects under 60 y, 0.48 was taken as the segregation point between slow and rapid phenotypes in the present study, such that subjects with a metabolite ratio <0.48 were classified as slow acetylators and those with a ratio >0.48 were classified as rapid acetylators. For subjects, >60 y, 0.44 was taken as the point to separate

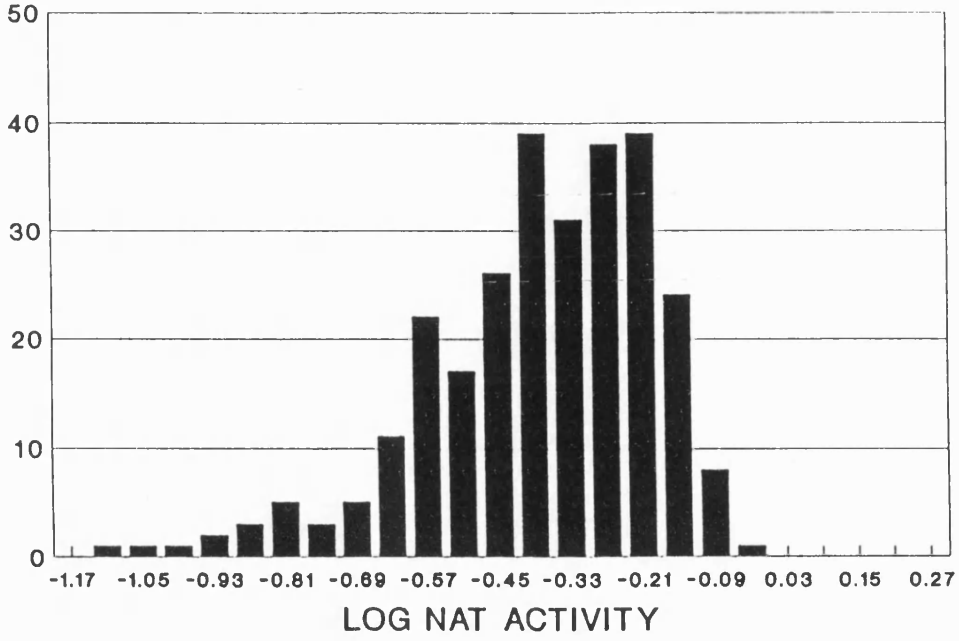


Figure 6.1.7 - Log frequency distribution histogram of NAT activity in 277 controls.

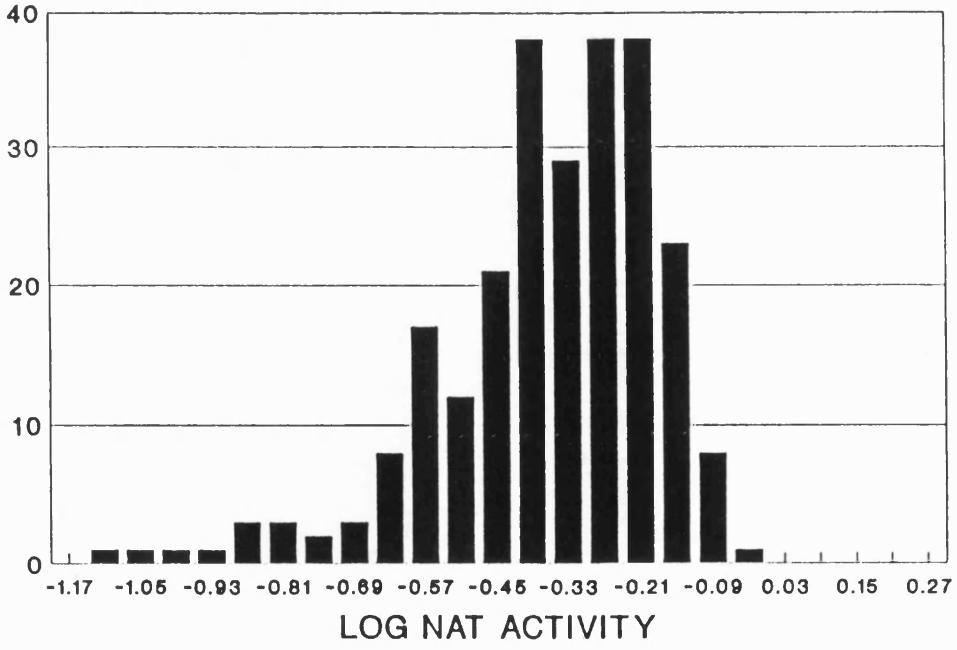


Figure 6.1.8 - Log frequency distribution histogram of NAT activity in 248 subjects, under 60 y of age.

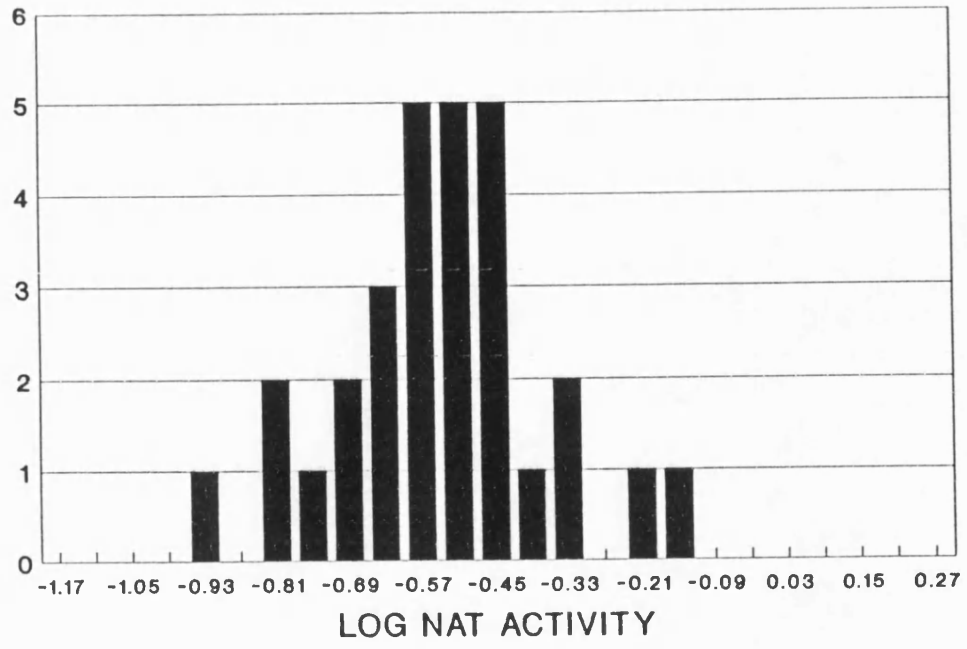


Figure 6.1.9 - Log frequency distribution histogram of NAT activity in 29 subjects, over 60 y of age.

Table 6.1.8 - The proportion of healthy subjects with slow and rapid NAT phenotypes

Group	NAT activity			
	Slow	%	Rapid	%
<60 y	134/248	(54.0%)	114/248	(46.0%)
>60 y	25/ 29	(86.2%)	4/ 29	(13.8%)

slow from rapid acetylators. The proportion of young and elderly subjects with slow and rapid phenotypes is shown in Table 6.1.8. The proportion of slow acetylators in elderly subjects (86.2%)

is highly significantly different from that in subjects under 60 y (54%) ($p<0.01$).

6.1.9 Discussion

Effect of Environmental Factors

The effect of age on NAT activity is subject to conflicting reports. In the present study, no correlation between subject age and NAT activity was found when a population of 277 healthy young and elderly subjects was examined. This is in agreement with several other workers who also used caffeine metabolite ratios to determine NAT activity (Grant et al, 1983b; Bartsch et al, 1990; Vineis et al, 1990). Age also had no effect on NAT activity determined with probe drugs other than caffeine (Evans et al, 1960; Farah et al, 1977; Clarke et al, 1982; Evans et al, 1983; Mommsen & Wolf, 1985; Philip et al, 1987a). No significant correlation between acetylation rate and age was found when slow and rapid phenotypes were examined separately and NAT activity was unaffected by age when the younger and elderly subjects were studied as separate groups, in agreement with Pontiroli et al (1985). However, NAT activity was significantly lower in the elderly (>60 y) than in subjects <60 y. Furthermore, age did seem to have a slight effect on the degree of NAT activity within the slow phenotype, such that NAT activity tended to decrease with increasing age. Iselius & Evans (1983) also found that acetylation capacity was affected by age, while Kergueris et al (1986) observed that isoniazid metabolism decreased with age in the slow phenotype but was unaffected by age in rapid acetylators.

The present study shows that 54% of healthy subjects <60 y are slow acetylators, compared to 86% of elderly subjects. Similarly, Gachalyi et al (1984) found there were 50% slow acetylators in 128 subjects <60 y and 66.4% slow acetylators in 125 subjects >60 y. Szorady et al (1987) found that 66% of elderly subjects (>60 y) were slow acetylators compared to 50% in the age range 19-59 y. 68% of a group of control subjects were found to be slow acetylators in the study by Lang et al (1986). Although they do not give the mean age of this group, one might presume that they are relatively elderly subjects as they are age-matched with a group of colorectal cancer patients. Ilett et al (1987) also found an excess of slow acetylators in old control subjects in comparison with younger controls. In contrast, Ladero et al (1991) and Philip et al (1987a) found that the distribution of the slow acetylator phenotype in healthy elderly subjects was around 60% and not different to that found in younger control groups. Evans et al (1989) used caffeine to determine acetylator phenotype in children and adolescents between the ages of 3 and 21 y and found that only 37.5% were slow acetylators. The reason for the apparent change in acetylation capacity with age is unknown.

As the antimode for the frequency distribution of NAT activity in elderly subjects was lower than that for younger subjects, one might conclude that there is some diminution in acetylating capacity with age. This observation is in agreement with several other studies although it would appear that any variation conferred by age is slight in comparison with that of genetic origin, in that a proportion of elderly subjects do exhibit rapid acetylation.

Body mass index was found to have no effect on NAT activity in either healthy young or elderly subjects. Grant et al (1983b) and Philip et al (1987a) also found that subject weight did not alter acetylation capacity in healthy volunteers.

NAT activity was not found to differ with subject gender in either elderly or younger volunteers, in agreement with several other workers, who have either used caffeine (Grant et al, 1983b; Tang et al, 1991) or other drugs to determine acetylator status (Cartwright et al, 1982; Evans et al, 1983; Miller & Cosgriff et al, 1983; Mommsen & Wolf, 1985; Ladero et al, 1991). In addition, acetylation capacity was not altered by OCS administration, in agreement with Hardy et al (1988) which indicates that while the cytochrome P450 enzymes may be susceptible to inhibition by steroids, NAT activity remains relatively unaltered.

NAT activity was unaffected by cigarette smoking in subjects <60 y. Similarly, acetylator phenotype was not influenced by cigarette smoking in the studies by Vineis et al (1990) and Bartsch et al (1990). Female smokers had a similar capacity for N-acetylation to nonsmokers, whether they were using OCS or not, which confirms the observation that NAT is a noninducible enzyme (Hein, 1988a). However, NAT activity in male smokers was less than in nonsmokers. Tang et al (1991) using caffeine as a metabolic probe for NAT activity also found that smokers within the slow phenotype had significantly lower NAT activity than nonsmokers, irrespective of the metabolite ratio used to determine acetylator phenotype. Furthermore, Cartwright et al

(1982) found that there was a slight excess of slow acetylators in smoking subjects compared to nonsmokers, although the frequencies between the two groups were not significantly different. The reason for these observations in younger subjects remains unclear but in elderly subjects NAT activity was unaffected by cigarette smoking whether males or females were studied. Other workers have also found that acetylation capacity was independent of smoking status (Cartwright et al, 1982; Evans et al, 1983; Miller & Cosgriff et al, 1983; Hardy et al, 1988).

Effect of Genetic Factors

The hepatic N-acetylation of arylamines is catalysed by acetylcoenzyme A (AcCoA)-dependent N-acetyltransferases which exhibits genetically mediated polymorphic expression in mammalian species (Evans et al, 1960; Weber & Hein, 1985; Hein, 1988a). This enzymatic biotransformation of aromatic amines is controlled by simple Mendelian inheritance of 2 major alleles at a single gene locus (Weber & Hein, 1985; Hein, 1988 a,b). Genetic studies in humans (Kilbane et al, 1990) provide strong evidence for codominant expression of these alleles which would account for the trimodal distribution of individuals into the rapid, intermediate and slow acetylator phenotypes, which have occaisionally been observed (Gascon et al, 1987; Tang et al, 1987; Kilbane et al, 1990).

As reviewed by Weber & Hein (1985) and Smith (1989), a large number of in vivo methods for determining acetylation phenotype exist. Phenotyping tests should be quick and easy to perform, not hazardous and give unequivocal classification of individual

phenotypes. These criteria have not been met by any one phenotyping test to date. All involve administration of a foreign chemical to subjects and most are unable to clearly determine individual phenotype (Evans, 1989). More accurate techniques for estimating acetylator phenotype are elaborate, involving collection of timed blood samples and can therefore only be performed in small groups of subjects. Recent work has shown that some of these problems may be overcome by using caffeine as a test drug to determine acetylation status (Rieder et al, 1991) and results from the present study suggest that caffeine might be used as a metabolic probe to phenotype subjects as rapid or slow N-acetylators.

NAT activity ranged 11-fold in 277 healthy subjects. Although different modes were apparent from the frequency distribution histogram of NAT activity, bimodality of the distribution was not obvious. However, logging the metabolite ratios resulted in a distribution that was likely to be bimodal in nature, with clearer separation between modes, in agreement with Jackson et al (1986). It may thus be possible to measure NAT activity and determine acetylator phenotype using the log metabolite ratio AAMU/(AAMU + 1-MU + 1-MX), in agreement with Bartsch et al (1990)

In addition to the differences in acetylation capacity observed between the elderly and subjects <60 y, the apparent antimodes in the frequency distributions for these two groups were slightly different. Whereas an antimode at a metabolite ratio of 0.48 appeared to separate slow from rapid acetylators in subjects <60 y, the equivalent antimode in elderly subjects was 0.44. As NAT activity seems to be reduced in old age, the same

phenotype separation criteria should not be applied to both groups (Evans, 1989). 54% of the healthy population <60 y were of the slow acetylator phenotype, while 46% were rapid acetylators. Similar frequencies of rapid and slow phenotypes were obtained in several other studies involving Caucasian volunteers (Grant et al, 1983a; Gascon et al, 1987; Tang et al, 1987; Kilbane et al, 1990). In contrast, 84% of elderly subjects >60 y were slow acetylators, which is slightly greater than the frequency of slow acetylators found by Gachalyi et al (1984) and Szorady et al (1987).

While a simple technique involving urine collection, such as the caffeine test may be less sensitive in determining acetylator phenotype than tests involving collection of several blood and urine samples, it may prove to be very useful for large population studies, as originally proposed by Grant et al (1984). The main problem with nearly all of the chemical tests performed to date is that they are only capable of phenotyping and not genotyping individuals. While some studies claim to distinguish the intermediate heterozygous acetylator phenotype (Tang et al, 1987; Kilbane et al, 1990; Irshaid et al, 1991), most methods do not allow such classification in vivo. In fact, most in vitro studies (Kirlin et al, 1989; Land et al, 1989), with the exception of that by Kirlin et al (1991), have also failed to separate rapid, intermediate and slow phenotypes. Tang et al (1987) and Kilbane et al (1990) both measured the AAMU/(AAMU + 1-MX + 1-MU) metabolite ratio to determine NAT activity and claimed that the caffeine test could be used to genotype individuals. This study, like most others was unable to confirm such results as only two phenotypes could be observed following

oral administration of caffeine. Tang et al (1987) assigned arbitary antimodes in the frequency distribution at 0.36 and 0.58, while Kilbane et al (1990) assigned antimodes at 0.38 and 0.66 to separate phenotypes. Although the present study has used the same metabolite ratio as these two groups, it is important that each study defines its own antimode, rather than take those assigned to frequency distributions in other studies because until the caffeine test is standardized, differences in methodology will inevitably result in variation between NAT activities determined in different laboratories.

The caffeine test for testing NAT phenotype is worthy of continued study in light of the fact that it requires oral intake of an innocuous compound for widespread use. Unlike tests using other probe drugs, it does not appear necessary to strictly monitor either the timing or the quantity of caffeine intake or urine collections during the test (Evans, 1989). Thus caffeine may permit the phenotyping and possibly genotyping of individuals for NAT with minimal inconvenience.

6.2 N-ACETYLTRANSFERASE ACTIVITY IN PATIENTS WITH FAP, COLORECTAL CARCINOMA AND OTHER NEOPLASTIC DISORDERS

6.2.1 Introduction

N-acetyltransferase is responsible for the metabolism of certain carcinogenic aromatic amines. While hepatic NAT activity is thought to represent a detoxification pathway (Beland & Kadlubar, 1986), an enzyme closely related to NAT in bowel tissue can activate arylamines into ultimate carcinogens that may bind to DNA (Flammang & Kadlubar, 1986). Since NAT is subject to genetic polymorphism (Evans et al, 1960), phenotypic variation in the activity of this enzyme may contribute to individual differences in susceptibility to arylamine-induced carcinogenesis.

6.2.2 Results

NAT activities in patients with colorectal cancer, leukaemia, carcinoma of the lung, head and neck and in patients with FAP are shown in Table 6.2.1. NAT activity of a group of 277 control volunteers is also shown.

6.2.3 Effect of Age

No correlation between subject age and NAT activity existed in 37 patients with FAP ($r=0.0853$; $p>0.5$) or in 16 of their relatives at risk for the disease ($r=0.3007$; $p>0.1$). In addition, age was found to have no effect on NAT activity in 12 patients with colorectal carcinoma ($r=0.0596$; $p>0.5$). This relationship was also found if the effect of age was studied for slow and rapid acetylator phenotypes separately.

Table 6.2.1 - NAT activity in patients with FAP, their relatives at risk for FAP, patients with neoplastic disease and control volunteers

Group	n	Mean activity ± SEM	Median	Range
FAP	37	0.324 ± 0.024	0.283	0.054 - 0.640
At Risk	16	0.414 ± 0.031	0.388	0.249 - 0.697
Colorectal cancer	12	0.329 ± 0.031	0.299	0.184 - 0.586
Lung, head & neck	51	0.405 ± 0.019	0.387	0.170 - 0.728
Leukaemia	54	0.400 ± 0.022	0.379	0.068 - 0.706
Controls	277	0.450 ± 0.010	0.443	0.077 - 0.886

The mean age of 37 patients with FAP was 36.38 ± 3.85 y and was not significantly different to the mean age of 36.62 ± 3.43 y in a group of 248 control volunteers under 60 y of age ($p>0.5$). The mean age of 16 relatives at risk for FAP was 31.25 ± 4.03 y and was also not significantly different from the mean age of 248 controls ($p>0.05$).

The mean age of 12 patients with cancer of the large bowel was not significantly different from the mean age of 72.9 ± 3.03 y in a group of 29 control subjects over 60 y ($p>0.1$).

The mean ages of 54 patients with leukaemia and 51 patients with carcinoma of the lung, head and neck were significantly lower ($p<0.001$ and $p<0.05$, respectively) than that of the elderly control group over 60 y old. Enzyme activities in patients with lung cancer and leukaemia were thus compared to both elderly

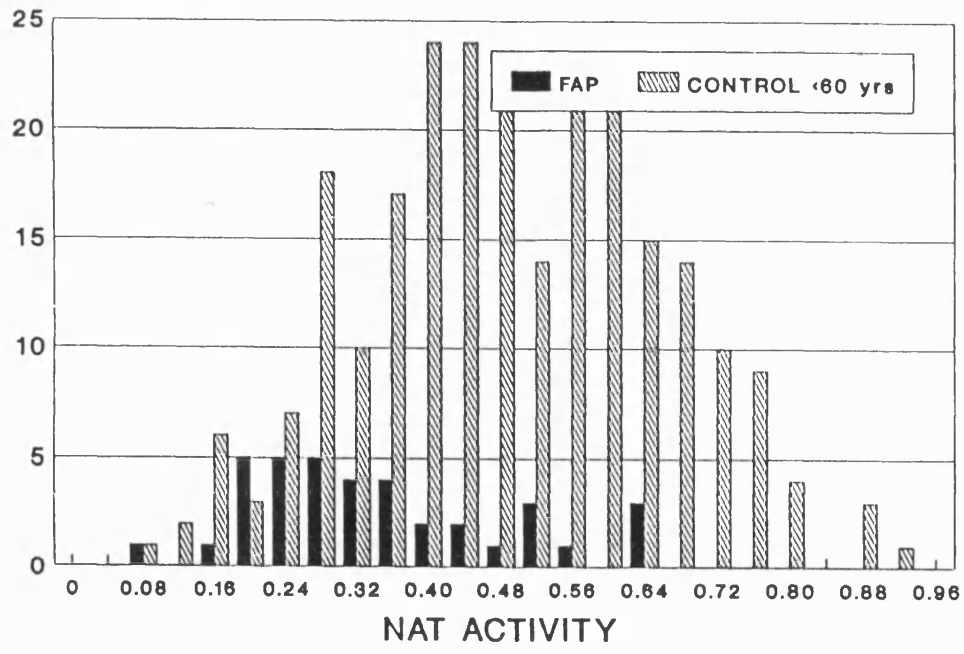


Figure 6.2.1 - NAT activity in patients with FAP, compared to age-matched controls.

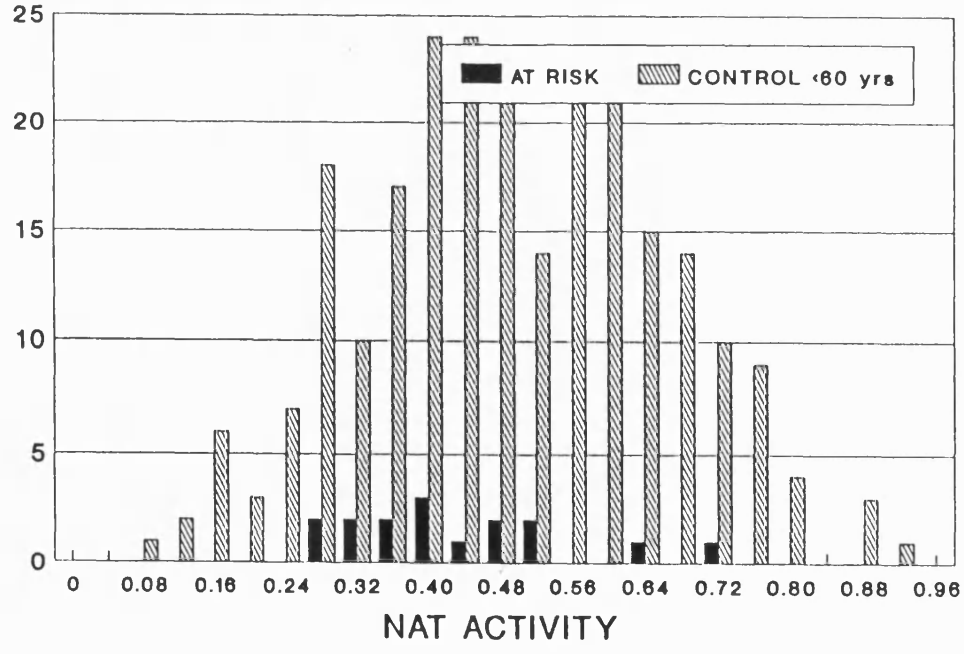


Figure 6.2.2 - NAT activity in relatives at risk for FAP compared to age-matched controls.

controls and the control group <60 y.

NAT activity in patients with FAP was highly significantly less than that of 248 age-matched controls ($p<0.000005$) (Fig.6.2.1) and less than that of the whole control population of 277 volunteers ($p<0.00005$).

NAT activity in relatives at risk for FAP was also less than that of 248 age-matched controls ($p<0.01$) (Fig.6.2.2) but not significantly different to that of the whole control population ($p>0.1$). NAT activity of FAP patients was significantly lower than that of their relatives at risk ($p<0.05$) (Fig.6.2.3).

NAT activities for patients with colorectal cancer, leukaemia and lung, head and neck cancer were compared to elderly age-matched and general population controls (Table 6.2.2).

Patients with colorectal cancer had a mean NAT activity that was not significantly different from that of elderly age-matched controls ($p>0.1$) (Fig.6.2.4). However, NAT activity in these patients was significantly less than that of 277 general population controls ($p <0.01$). NAT activity in 51 patients with carcinoma of the lung, head and neck was significantly higher than that of elderly controls ($p<0.005$) but similar to that of the whole population ($p=0.05$). NAT activity in 54 patients with leukaemia was also higher than that of elderly controls ($p<0.05$) but slightly less than that of the whole population ($p=0.04$).

The group of patients with FAP had lower mean NAT activity than the patients with leukaemia ($p<0.05$) or carcinoma of the lung, head and neck ($p<0.01$). NAT activity in patients with FAP was not significantly different from that in patients with colorectal cancer ($p>0.5$).

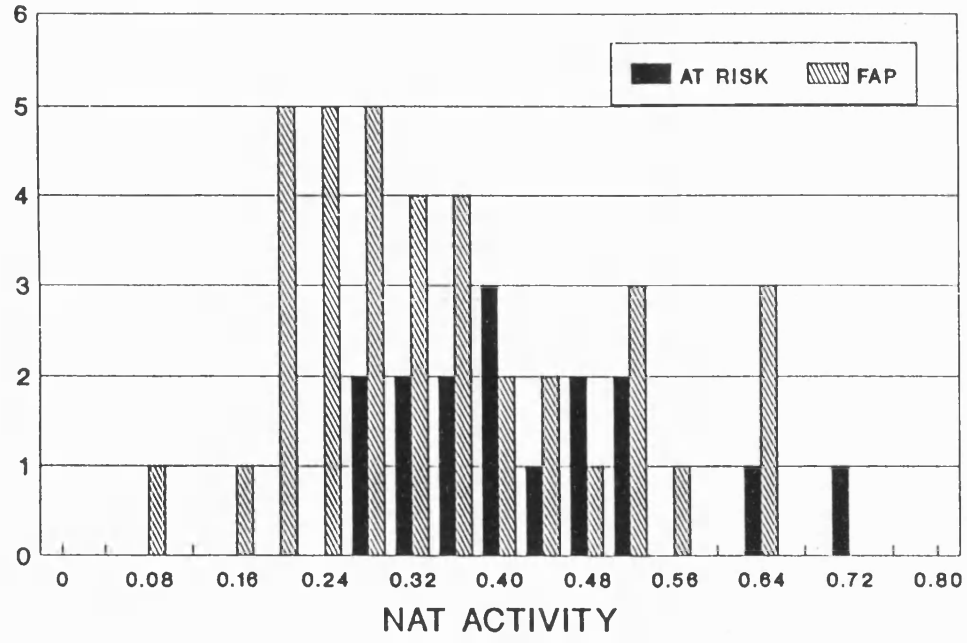


Figure 6.2.3 - NAT activity in patients with FAP, compared to their relatives at risk for FAP.

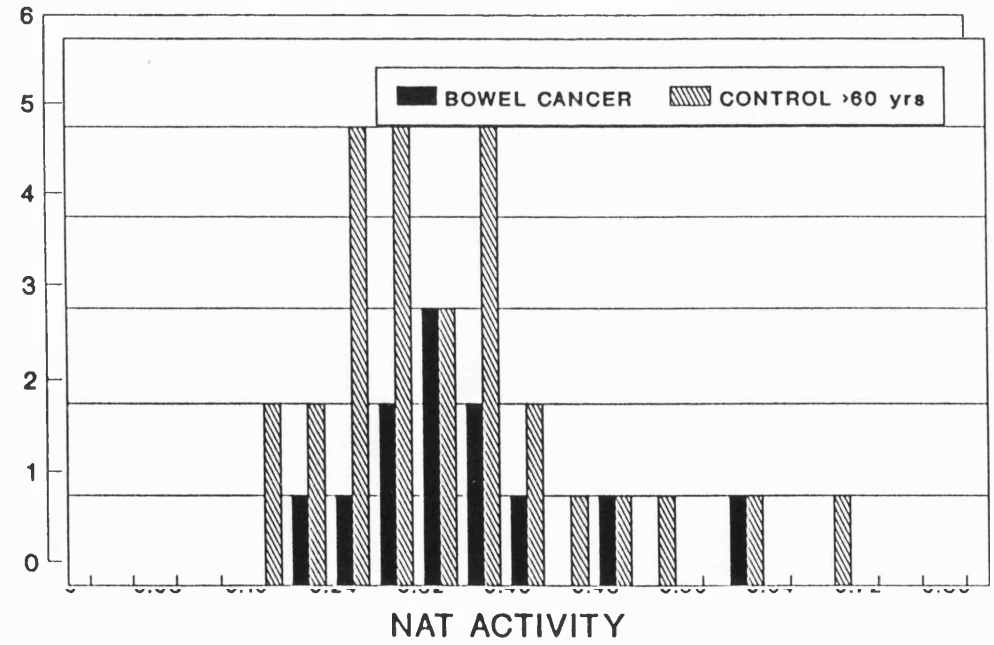


Figure 6.2.4 - NAT activity in patients with colorectal cancer, compared to age-matched controls.

6.2.4 Genetic Control of N-Acetyltransferase Activity

The frequency distribution histograms for NAT activity in 37 patients with FAP and 16 of their relatives at risk for FAP are depicted in Figs.6.2.5 and 6.2.6, respectively. The proportion of these patients and their relatives with slow NAT activity (ratio <0.48) is shown in Table 6.2.3.

Table 6.2.3 - The proportion of FAP patients and their relatives at risk for FAP with slow and rapid NAT phenotypes

Group	NAT activity			
	Slow	%	Rapid	%
FAP	29/ 37	(78.4%)	8/ 37	(21.6%)
Risk	10/ 16	(62.5%)	6/ 16	(37.5%)
Controls				
-age-matched	134/248	(54.0%)	114/248	(46.0%)
-all	144/277	(52.0%)	133/248	(48.0%)

78.4% of the FAP patients were of the slow NAT phenotype (Fig.6.2.5). This was significantly greater than the proportion of age-matched controls with slow NAT activity ($p<0.01$) and also significantly greater than the proportion of slow acetylators in 277 general population controls ($p<0.01$).

62.5% of the group at risk for FAP were of the slow NAT phenotype (Fig.6.2.6) which was not significantly different from number of age-matched controls ($p>0.05$) and general population controls ($p>0.05$) with slow NAT activity. The proportion of slow

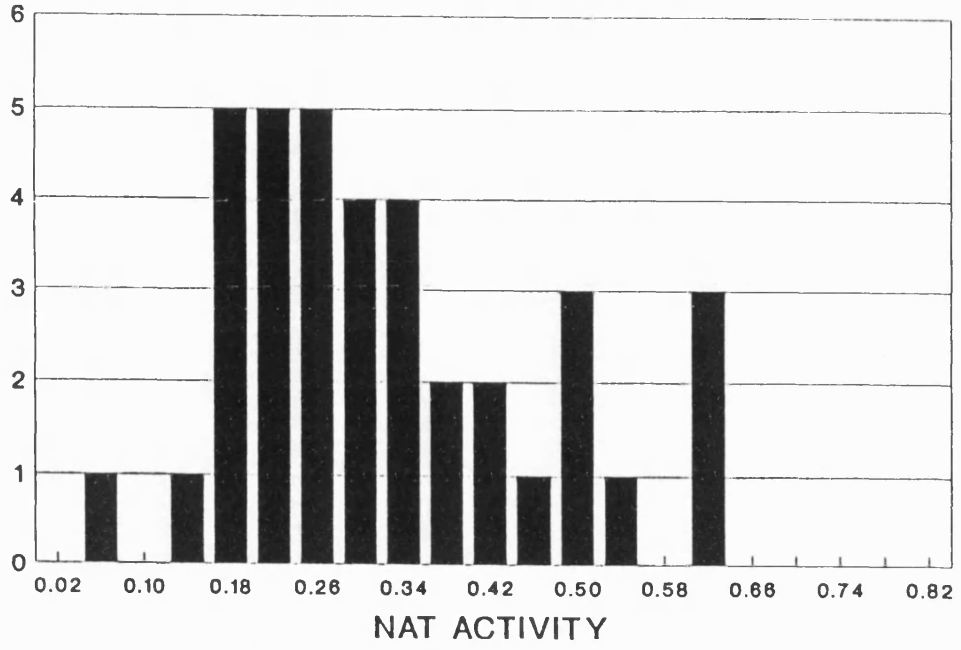


Figure 6.2.5 - Frequency distribution histogram of NAT activity in 37 patients with FAP.

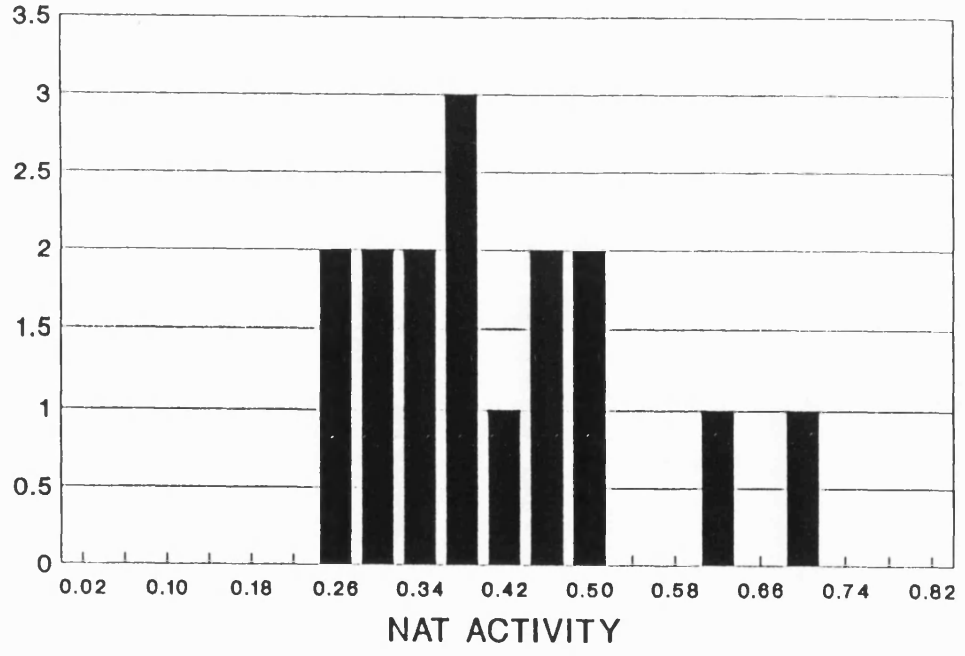


Figure 6.2.6 - Frequency distribution histogram of NAT activity in 16 relatives at risk for FAP.

acetylators in patients with FAP was not significantly greater than that of their relatives at risk for FAP ($p>0.05$).
The proportion of 35 FAP patients with high P450IA2 activity and slow NAT activity was also calculated (Table 6.2.4) and compared to that of 226 age-matched controls (Table 6.2.5).

Table 6.2.4 - The proportion of FAP patients with slow NAT and high P450IA2 activity

	P450IA2 activity			
Acetylation phenotype	High	%	Low	%
Slow	9/35	(25.7%)	20/35	(57.1%)
Fast	1/35	(2.9%)	5/35	(14.3%)

Table 6.2.5 - The proportion of healthy controls <60 y with slow NAT and high P450IA2 activity

	P450IA2 activity			
Acetylation phenotype	High	%	Low	%
Slow	16/226	(7.1%)	99/226	(43.8%)
Fast	22/226	(9.7%)	89/226	(38.4%)

The proportion of FAP patients with both slow NAT activity and high P450IA2 activity is 25.7% in contrast to the proportion of 7.1% in age-matched controls. While the proportion of subjects

with slow NAT and slow P450IA2 activities is similar for both groups studied, it appears that the number of fast acetylators is considerably reduced in FAP patients compared to age-matched controls.

The frequency distributions of NAT activity for patients with colorectal cancer, carcinoma of the lung, head and neck and leukaemia are shown in Figs.6.2.7, 6.2.8 and 6.2.9, respectively. The proportion of these patients with the slow NAT phenotype (ratio <0.44) is shown in Table 6.2.6.

Table 6.2.6 - The proportion of patients with colorectal cancer, leukaemia and carcinoma of the lung, head and neck with slow and rapid NAT phenotypes

Group	NAT activity			
	Slow	%	Rapid	%
Colorectal	10/ 12	(83.3%)	2/ 12	(16.7%)
Leukaemia	31/ 54	(57.4%)	23/ 54	(42.6%)
Lung,head & neck	29/ 51	(56.9%)	22/ 51	(43.1%)
Controls				
-elderly	25/ 29	(86.2%)	4/ 29	(13.8%)
-all	144/277	(52.0%)	133/248	(48.0%)

83.3% of the patients with colorectal cancer were of the slow NAT phenotype (Fig.6.2.7). This was not significantly different from the proportion of slow acetylators in a group of elderly age-matched controls ($p>0.05$) but significantly different from

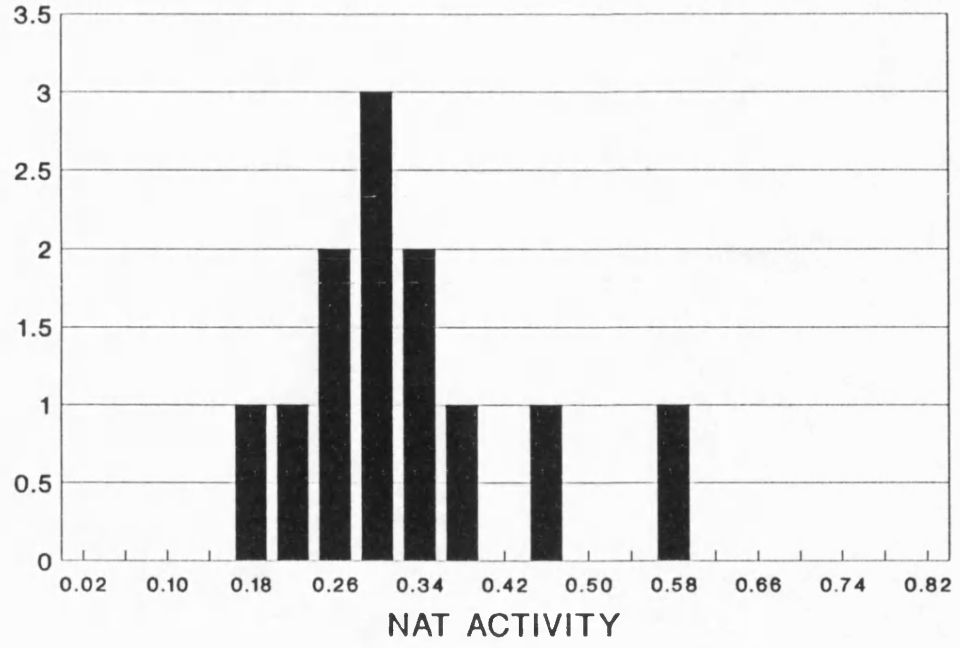


Figure 6.2.7 - Frequency distribution histogram of NAT activity in 12 patients with colorectal cancer.

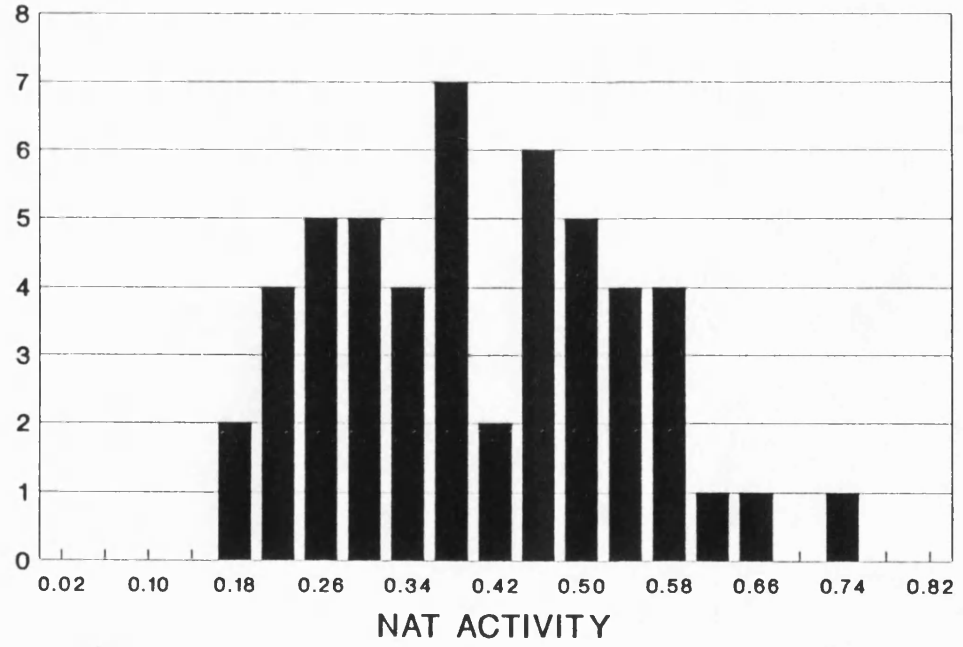


Figure 6.2.8 - Frequency distribution histogram of NAT activity in 51 patients with carcinoma of the lung, head and neck.

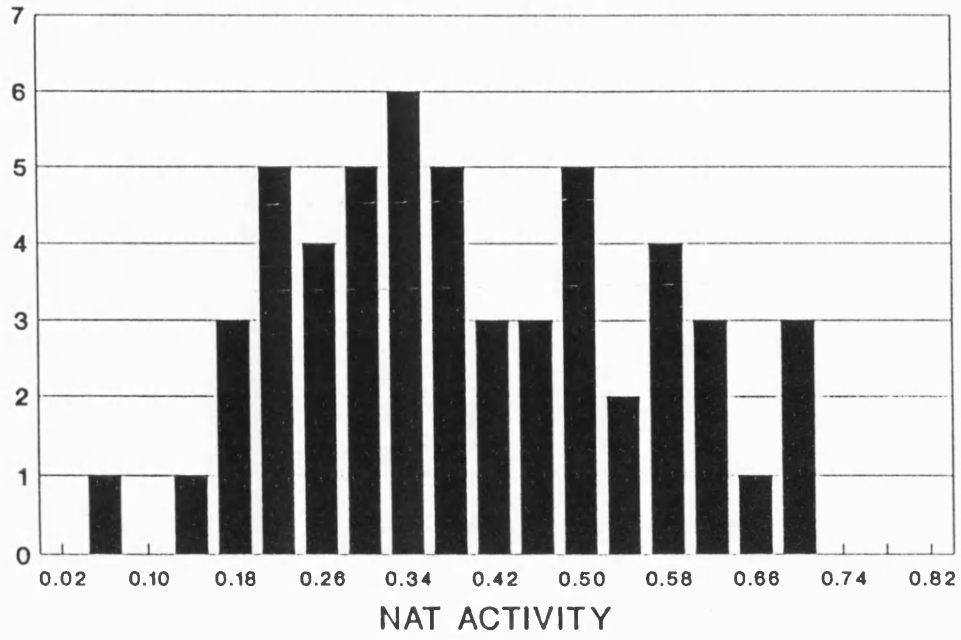


Figure 6.2.9 - Frequency distribution histogram of NAT activity in 54 patients with leukaemia.

the proportion of 277 general population controls with slow NAT activity ($p<0.05$). The proportion of slow acetylators in patients with colorectal cancer was not significantly different from that in patients with FAP ($p>0.05$).

56.9% of patients with carcinoma of the lung, head and neck had slow NAT activity (Fig.6.2.8) which is significantly lower than the proportion of slow acetylators in elderly control subjects ($p<0.01$) but not significantly different from that of the general population ($p >0.05$). The proportion of lung cancer patients with slow acetylation phenotype was significantly less than that in patients with FAP ($p<0.05$).

Likewise, 57.4% of patients with leukaemia were slow acetylators (Fig.6.2.9) which was significantly lower than the proportion of

elderly control subjects with slow NAT activity ($p<0.01$) but not significantly different from general population controls ($p>0.05$). The proportion of slow acetylators in patients with leukaemia was significantly less than that in patients with FAP ($p<0.05$).

6.2.5 Discussion

The present study shows that a much greater proportion of patients with FAP were slow acetylators compared to control subjects. Relatives of the FAP patients, who are considered to be at risk for the disease also had lower mean NAT activity when compared to age-matched controls, although the proportion of slow acetylators in this group of relatives was not different to that of healthy subjects. The difference in proportion of acetylator phenotypes observed in patients with FAP may alter the way in which these patients metabolise carcinogenic arylamines.

N-OH-arylamines are recognised as proximate carcinogens and are generally more carcinogenic than their parent amines (Schut & Castonguay, 1984). Conversion of these proximate carcinogens to ultimate carcinogens, that bind to DNA, is thought to involve several acyltransferases (Schut & Castonguay, 1984; Kadlubar & Hammons, 1987). Hepatic NAT activity is thought to represent a detoxification pathway (Poupko et al, 1979; Beland & Kadlubar, 1986). As patients with FAP tend to be slow acetylators, it is possible that they are less able to N-acetylate arylamines and detoxify activated N-OH arylamines in the liver. However, O-acetyltransferase (OAT) which is closely related to NAT (Flammang et al, 1985; Hein, 1988a; Land et al, 1989; Kirlin et

al, 1989) is capable of activating N-OH arylamines into ultimate carcinogens (Flammang & Kadlubar, 1986). For species susceptible to arylamine-induced colon cancer, it has been proposed that biliary excretion of the N-OH-arylamine N-glucuronide (Poupko et al, 1979; Meerman et al, 1982), its deconjugation by bacterial b-glucuronidases and conversion of the N-OH-arylamine to an ultimate carcinogen, within the intestinal epithelium are critical steps leading to the initiation of tumorigenesis (Kadlubar & Beland, 1985). The glucuronidation of N-OH arylamines may be prevented by prior hepatic N-acetylation of the aromatic amine and thus N-acetylation of either the arylamine or the N-OH-arylamine is considered to be a detoxification step (King & Glowinsky, 1983). Conversely, if the arylamine is initially N-hydroxylated, further activation may occur through O-acetylation. The pathway that predominates will therefore depend on levels of cytochrome P450, in addition to NAT (Beland & Kadlubar, 1986).

As NAT activity in bladder (Kirlin et al, 1989) and liver (Land et al, 1989) is much greater than OAT activity, for either slow or rapid acetylators, OAT mediated activation is probably minor in comparison to NAT-mediated detoxification of arylamines (Kirlin et al, 1989). This would also seem likely in view of the fact that the arylamine-DNA adducts in target tissues are non-acetylated derivatives (Beland et al, 1983; Kadlubar & Beland, 1985).

Results from the current study also suggest that the detoxification process involving hepatic NAT is more important than OAT activity in bowel tissue, as far more of FAP patients, who are at a high risk for developing bowel cancer, were slow

acetylators. As a greater proportion of the FAP patients had high P450IA2 activity when compared to healthy subjects, in the present study, this strongly suggests that a large degree of activation of arylamines by P450IA2 and a low degree of detoxification of arylamines by NAT is occuring in FAP.

The importance of NAT activity can be illustrated by the fact that tissue susceptibility to N-OH-arylamines in the dog, can be correlated with the NAT enzyme system. For example, the administration of several arylamines to dogs leads to urinary bladder tumours, even though the dog has a total functional deficiency in the capacity to N-acetylate arylamines. These observations indicate that N-acetylation is not involved in arylamine activation in bladder carcinogenesis. The dog's inability to detoxify arylamines by NAT partly explains the high rate of arylamine-induced bladder cancer in this species (Lower, 1982).

The fact that the present study recorded a greater proportion of slow acetylators in a group of patients with FAP is not unusual. Patients with bladder cancer are more commonly slow acetylators (Cartwright et al, 1982; Evans et al, 1983; Hanssen et al, 1985; Ladero et al, 1985; Vineis et al, 1990) with high P450IA2 activity (Bartsch et al, 1990). The highest arylamine-haemoglobin adducts measured in the study by Bartsch et al (1990) were found in the "slow-acetylator and fast-oxidiser" group of subjects. Lowest adduct concentrations were found in the "fast-acetylator and slow-oxidiser" group. It is proposed that individuals at risk for bladder cancer are less able to detoxify carcinogens that are activated hepatically by P450IA2, as they have the slow NAT phenotype and are more prone to

bladder cancer than rapid acetylators (Hein, 1988a). The present results suggest that a similar situation exists for patients with FAP, in that a greater proportion of these patients have high P450IA2 activity and slow NAT activity than healthy controls. It is tempting to speculate that the relatives at risk for FAP with the slow- acetylation and fast-oxidation phenotypes are more susceptible to developing FAP. Thus, determining both phenotypes may allow a better prediction of the risk of FAP and bladder cancer, than measuring NAT phenotype alone. These findings support the role of NAT activity in the detoxification, and P450IA2 in the activation of arylamines.

DNA adducts have been found to be distributed in the same pattern as foregut tumours in patients with FAP and such adducts are present in significantly greater amounts in these patients compared to controls (Spigelman et al, 1991). Bile from patients with FAP was found to impart a greater adduct load on small bowel target mucosa than bile from control subjects, implying that the excess of DNA adducts detected in FAP mucosa resulted from carcinogens transported in bile (Spigelman et al, 1991). The present study suggests that the observed difference of DNA adducts and tumours in the forgut mucosa of patients with FAP may be partially attributed to the defective detoxification of carcinogens by NAT in the liver.

NAT activity and the frequency of slow acetylators in patients with colorectal cancer was not significantly different from that measured in an age-matched control group. As patients with this type of cancer tend to have a higher mean age than control subjects (Cartwright et al, 1982; Ladero et al, 1985;

Moller-Jensen, 1983), it is essential that enzyme activities in patients are compared to age-matched controls. When the 12 patients with colorectal carcinoma were compared to 277 general population controls, there was a highly significant over-representation of slow acetylators in the patient group, in contrast to an equal representation of slow acetylators between patients and age-matched controls.

P450IA2 activity in these patients was not different from that of healthy subjects. Taken together, these findings suggest that arylamine metabolism is not altered in colorectal cancer. This observation is contradictory to two studies which claimed that rapid acetylators were more prone to bowel cancer than control subjects (Lang et al, 1986; Ilett et al, 1987). There are several possible reasons for the disparity in results between our study and those by other groups.

Firstly, caffeine metabolite ratios were used in the present study to determine NAT activity, whereas Ilett et al (1986) and Lang et al (1987) used sulphamethazine to determine acetylation phenotype. In theory, this should not account for the difference in results, as complete concordance between caffeine and sulphamethazine tests has been shown (Tang et al, 1991). However, in patients with neoplastic disease, it is possible that hypoalbuminaemia may be present (Webster et al, 1990). As sulphamethazine is highly bound to plasma albumin, the plasma concentrations may be altered in patients with cancer unless their liver function is normal. Neither of the studies listed above report on patients liver or kidney function, whereas the present work only studied patients with liver and kidney function values within the normal range.

Secondly, it is essential that the ethnic composition of each group being studied is known (Evans, 1984) as the distribution of phenotypes varies in different ethnic and geographic populations (Weber & Hein, 1985; Evans, 1989). This study therefore included Caucasian subjects only. The other studies however, either failed to define the ethnic origin of their case and control groups, or included subjects of varying ethnic origin in their study, such as Hispanics, Orientals and American white and blacks. Whereas around 50% of Caucasian populations are slow acetylators, only 10% of the Japanese (Grant et al, 1983 a,b; Deguchi et al, 1990) and 5% of Eskimos are slow acetylators, while 68% of Arabians (Irshaid et al, 1991), 83% of Egyptians and 90% of Moroccans are slow acetylators (Uetrecht & Woosley, 1981; Weber & Hein, 1985). While NAT phenotypes are presumed to be similar in frequency between white and black Americans (Evans et al, 1960), Relling et al (1991) found a greater prevalence of fast acetylators among American black compared to white children. In addition, Tang et al (1991) showed that Oriental slow acetylators had lower NAT activity than slow white acetylators. Therefore, data from studies including American blacks, Orientals and Europeans should be interpreted with caution, particularly when the study is investigating cancer incidence, since individual susceptibility for different cancers varies remarkably with ethnic and geographic location (Polednak, 1989).

Before any association between acetylator phenotype and a disorder can be made, satisfactory control groups are required (Evans, 1984). Patient groups in the present studies were compared to general population controls. Other patients who do

not suffer from the disease being investigated are not considered to be good controls (Evans, 1984). The illness of the control patients used in some studies (Lang et al, 1986; Ilett et al, 1987) may have some relationship to the NAT polymorphism or the disease condition being investigated may interfere with the phenotyping test. There is a possibility that acetylation capacity is increased in neoplastic disease (Evans, 1984) which may explain the over-representation of rapid acetylators in colorectal cancer patients found in some studies.

Although patients with bowel cancer had undergone colectomy, as in the other studies, it seems unlikely that colectomy altered the phenotyping test. Caffeine is almost exclusively hepatically metabolised (Bonati et al, 1982) and it is NAT activity in this organ that has been measured in the present study. Certainly no significant correlation between the time of tumour resection and acetylation phenotype was found in the study by Ilett et al (1987). This is to be expected because NAT is an inherited, non-inducible phenomenen. Moreover, duodenal tumours may occur both before and after colectomy in patients with FAP, suggesting that the metabolic abnormality responsible for the putative excretion of carcinogens into bile is not greatly influenced by this operation.

The present study has found that patients with colorectal cancer have less acetylation capacity than healthy controls but similar acetylation capacity to healthy elderly, age-matched volunteers. Circumstantial evidence against rapid acetylation predisposing to bowel cancer is the fact that in Japan, where 90% of the population are rapid acetylators (Grant et al, 1983 a,b; Deguchi et al, 1990), the incidence of colorectal carcinoma is very low

(Moller-Jensen, 1983; Polednak, 1989). Also, an animal model for large bowel cancer utilises the rat, an animal which does not exhibit the acetylation polymorphism as all exhibit slow acetylation.

As the present study involves only 12 colorectal cancer patients, a number of well controlled studies are clearly needed to determine the role of NAT phenotype in bowel carcinogenesis. The role of acyltransferases in activation and deactivation of arylamines is complex, as is the relative contribution of hepatic and extrahepatic mechanisms in the process leading to cancer initiation.

The proportion of patients with leukaemia and carcinoma of the lung, head and neck who were slow acetylators was not significantly different to that in a group of general population controls. The fact that fewer of these cancer patients had slow NAT activity compared to elderly controls is probably explained by the fact that acetylation capacity may be reduced with age. Philip et al (1987b) also found no association between NAT phenotype and the incidence of lymphoma in 101 white Caucasian subjects. Drozdz et al (1987) found that 83% of patients with carcinoma of the larynx were slow acetylators compared to 60% of control subjects, even though the patients were under 60 years old and age was not significantly different between the two groups. Ritter et al (1986) however, found that the distribution of acetylator phenotypes in patients with cancer of the larynx and pharynx was similar to that of controls. The fact that bronchial cancer does not appear to affect subject phenotype in the present study, is in agreement with several other studies

(Burgess & Trafford, 1985; Philip et al, 1988; Roots et al, 1988).

Few of the enzymes involved in arylamine metabolism (NAT, P450IA2, glutathione transferase) show any type of co-ordinate regulation with each other in man (Ketterer et al, 1991). Thus, in pathways that involve catalysis by several different enzymes, one would expect to see several independent variations in the human population, interacting with each other. It is possible that a relationship between risk factors, such as NAT and P450IA2 phenotypes and macromolecular bound carcinogens derived from environmental exposure exist in FAP. A similar relationship between genetics and the environment is thought to exist in lung cancer aetiology, although the carcinogens are likely to be different for FAP as it is not thought to be a disease related to cigarette smoke.

CHAPTER SEVEN

GENERAL DISCUSSION

The present studies were undertaken to investigate the potential of caffeine as an *in vivo* probe for hepatic enzyme activities in man. Sensitive analytical techniques were developed to allow accurate quantitation of caffeine and its metabolites in urine and to demonstrate the *in vitro* biotransformation of caffeine and paraxanthine by human cDNA expressed cytochrome P450IA1.

The urinary caffeine test, involving measurement of caffeine metabolite ratios was developed to estimate the activities of the P450IA isozymes and N-acetyltransferase, which may be of toxicological significance in the pathogenesis of chemically-induced cancers (Vineis *et al*, 1990; Kawajiri *et al*, 1991).

The complexity of caffeine biotransformation is well documented (Bonati *et al*, 1982; Tang *et al*, 1983; Arnaud, 1984) with the potential for production of many urinary metabolites arising via combinations of N-demethylation, 8-hydroxylation and ring cleavage reactions. Difficulties to be encountered involve the potential for overlapping pathways of formation of a given caffeine metabolite, each of which may be mediated by different enzymes to varying degrees. In addition, the finding that polymorphic liver N-acetyltransferase mediates the production of yet another caffeine metabolite means that it is necessary to quantify AFMU, a polar and chemically labile compound in order to estimate the relative importance of the caffeine pathways (Callahan *et al*, 1983). Nonetheless, accurate analytical determination of AAMU, the deformylated metabolite of AFMU can be accomplished such that an index of acetylator phenotype can be determined in man (Section 2.6.3).

Considerable interindividual differences in P450IA activity were found in a healthy population, which reflect at least in part variation in genetic constitution as the distribution of activity within the population appeared to be bimodal (Section 5.1). As P450IA activity was higher in smoking subjects and in patients with carcinoma of the lung, head or neck compared to healthy nonsmokers, it would appear that the caffeine test is sensitive to induction by a component(s) of cigarette smoke and that P450IA activity, determined by the caffeine test may be of value as a marker for detection of susceptibility for lung, head or neck cancer (Section 5.2).

As P450IA2 activity is also subject to wide interindividual variation (Section 5.1) and is capable of activating aromatic amines (Nebert, 1991), identification of persons within the population with high P450IA2 activity may be important in determining susceptibility for aromatic amine-induced cancers. The observed increase in P450IA2 activity in patients with FAP (Section 5.4) may be due to induction by aromatic amines and may be involved in the development of FAP. In addition, the finding that more patients with FAP and colorectal carcinoma are of the slow acetylation phenotype than healthy subjects, such that fewer of these patients may be able to inactivate potential carcinogenic aromatic amines, further supports the involvement of polymorphic enzymes in chemically-induced diseases (Section 6.2).

In conclusion, results from the present study indicate that caffeine is of potential value as a probe for P450IA isozymes and N-acetyltransferase activities. In addition to detection of enzyme induction by environmental agents, such as cigarette

smoke, the caffeine test may be of clinical value in determining those individuals who are at risk for certain chemically-induced cancers.

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Appendix 1a -Effect of caffeine consumption on urinary metabolite profiles
1 CCB: 0-8 h urine

Vol		Age	Sex	Weight (kg)	Height (cm)	Smoker	OCS	Urine vol (ml)	Amount excreted in urine 17-DMX (mg)	17-DMU	1-MX	1-MU	AAMU	CAFF
#1	AS	22	F	63.6	178	-	Y	1260	3.301	0.768	11.8	19.17	39.1	2.431
#2	SF	27	F	73.1	173	-	Y	636	5.062	3.631	13.6	25.85	46.4	3.008
#3	SE	20	F	59.5	165	+		380	2.789	1.086	12.2	16.19	23.8	0.133
#5	FM	22	F	69.9	163	+	Y	505	1.479	0.404	15.3	28.29	25.5	0.717
#6	BB	28	M	91.3	193	-		605	2.916	1.240	9.94	21.93	31.8	0.471
#7	RB	32	M	70	178	-		475	4.322	1.111	8.27	19.87	44.1	0.313
#8	DS	54	F	64.5	165	-		535	5.098	0.946	7.35	25.81	60.9	0.625
#9	SO	22	F	57.2	168	+	Y	239	2.970	0.738	15.7	24.66	50.7	0.380
#10	EL	34	F	50	165	-		535	1.706	0.171	9.98	17.72	27.4	0.374
#11	JM	28	F	52.7	174	-		473	2.511	0.856	9.26	8.551	25.8	0.614
#12	JK	44	F	63.6	168	-		775	5.138	2.022	24.1	59.18	190.	0.550
#13	PL	23	M	54	178	-		383	4.703	0.543	12.6	9.479	38.1	1.206
#14	MB	30	M	82.6	183	+		250	5.455	0.752	25.8	36.14	21.5	0.367
#15	MH	31	M	66.7	165	-		870	6.96	1.096	5.88	16.05	76.0	1.827
#16	HH	24	F	60.4	168	-	Y	512	4.449	1.454	11.0	13.83	25.2	2.135
#17	CP	23	M	63.6	169	+		360	2.350	0.507	8.27	8.614	17.3	0.118
#18	SA	34	M	73.1	178	-		495	6.301	1.148	26.4	24.77	48.8	0.455

Appendix 1b -Effect of caffeine consumption on urinary metabolite profiles

1CCB: 0-8 h urine

Vol		Age	Sex	Weight (kg)	Height (cm)	Smoker	OCS	Urine vol. (ml)	Amount excreted in urine (mg)					
									17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#2	SF	27	F	73.1	173	-	Y	500	4.115	1.095	6.65	12.87	32.9	0.495
#3	SE	20	F	59.5	165	+		627	1.435	0.332	11.4	14.00	20.8	0.062
#6	BB	28	M	91.3	193	-		440	2.745	1.328	15.9	16.77	23.9	0.374
#7	RB	32	M	70	178	-		555	3.313	0.610	3.90	9.906	28.6	0.516
#8	DS	54	F	64.5	165	-		503	2.625	0.477	6.82	19.16	36.3	0.150
#9	SO	22	F	57.2	168	+	Y	369	5.826	1.055	23.6	38.37	99.7	1.051
#12	JK	44	F	63.6	168	-		775	2.945	0.627	12.1	29.68	88.6	0.193
#13	PL	23	M	54	178	-		432	5.374	0.760	10.6	8.959	27.3	0.855
#16	HH	24	F	60.4	168	-	Y	266	2.189	0.928	10.1	12.16	13.9	0.585
#17	CP	23	M	63.6	169	+		785	3.336	0.447	19.3	23.33	33.3	0.423

Appendix 1c -Effect of caffeine consumption on urinary metabolite profiles
4 CCBs: 0-8 h urine.

Vol		Age	Sex	Weight (kg)	Height (cm)	Smoker	OCS	Urine vol (ml)	Amount excreted in urine (mg)					
									17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#2	SF	27	F	73.1	173	-	Y	1089	4.072	0.794	5.21	14.20	26.5	0.522
#3	SE	20	F	59.5	165	+		400	4.012	0.568	11.1	16.52	21.4	0.116
#7	RB	32	M	70	178	-		390	7.062	1.298	10.3	24.16	53.0	0.897
#8	DS	54	F	64.5	165	-		618	5.654	1.341	13.4	37.76	60.1	0.729
#9	SO	22	F	57.2	168	+	Y	525	6.856	1.606	21.7	33.36	90.1	1.701
#11	JM	28	F	52.7	174	-		275	2.868	0.836	12.6	11.19	32.6	0.343
#12	JK	44	F	63.6	168	-		495	2.192	0.970	10.0	25.81	82.7	0.311
#13	PL	23	M	54	178	-		385	15.04	2.225	17.2	13.81	52.7	1.978
#14	MB	30	M	82.6	183	+		415	4.872	1.477	30.5	35.44	29.5	1.506
#15	MH	31	M	66.7	165	-		690	7.410	0.903	12.9	34.90	56.9	0.227
#16	HH	24	F	60.4	168	-	Y	315	12.72	1.335	23.4	29.28	36.8	0.973
#17	CP	23	M	63.6	169	+		555	6.676	0.388	20.4	37.00	46.6	1.287
#18	SA	34	M	73.1	178	-		1050	11.23	1.879	29.4	35.51	103.	2.436
#19	SH	50	F	62.3	161	-		775	9.462	1.154	18.1	28.00	77.5	2.821
#60	AG	25	M	82.8	184	-		860	12.04	0.928	28.9	34.90	34.4	0.507
#61	DB	39	F	63.2	168	+		180	6.951	2.608	30.3	23.01	14.4	0.775
#70	PB	23	M	66.7	165	-		330	7.748	1.296	39.9	7.923	107.	0.412
#71	YK	26	F	60.4	162	-		375	4.935	0.57	5.94	6.975	39.8	0.663
#86	HB	34	F	64.5	165	-		400	3.376	0.468	5.86	6.188	13.1	0.052

Appendix 1d -Effect of caffeine consumption on urinary metabolite profiles

1 CCB: 2-6 h urine.

Vol		Age	Sex	Weight (kg)	Height (cm)	Smoker	OCS	Urine vol (ml)	Amount excreted in urine (mg) 17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#2	SF	27	F	73.1	173	-	Y	120	1.852	0.201	4.85	9.716	11.9	0.813
#4	-AT	28	F	60.3	161	-		85	0.821	0.041	0.90	2.106	10.7	0.048
#7	RB	32	M	70	178	-		115	1.420	0.194	2.79	5.874	16.4	0.570
#9	SO	22	F	57.2	168	+	Y	240	3.372	0.343	12.7	9.837	41.4	0.794
#11	JM	28	F	52.7	174	-		70	1.204	0.645	3.60	3.670	7.80	0.379
#12	JK	44	F	63.6	168	-		205	1.582	0.576	6.69	16.38	55.6	0.444
#13	PL	23	M	54	178	-		120	3.008	0.471	3.01	2.409	8.85	0.709
#14	MB	30	M	82.6	183	+		96	1.392	0.133	5.34	8.4	5.83	0.348
#15	MH	31	M	66.7	165	-		195	2.154	0.265	4.30	12.77	19.6	0.520
#16	HH	24	F	60.4	168	-	Y	120	2.428	0.331	4.5	5.496	7.84	1.034
#17	CP	23	M	63.6	169	+		50	0.433	0.069	1.88	4.61	4.58	0.207
#18	SA	34	M	73.1	178	-		130	2.853	0.240	5.49	8.591	27.8	0.473
#19	SH	50	F	57.2	152	-		120	2.109	0.148	3.36	5.102	12.5	0.738
#60	AG	26	M	79.4	183	-		39	0.929	0.053	1.65	2.110	7.12	0.084
#61	DB	38	F	63.6	168	+		130	3.399	0.299	9.43	11.42	10.2	0.208
#70	PB	23	M	66.2	185	-		110	1.837	0.217	7.13	7.568	24.9	0.269
#71	YK	32	F	52	160	-		79	1.087	0.105	1.35	1.899	13.0	0.255
#86	HB	39	F	61	165	-		150	4.000	0.306	5.53	11.30	16.3	0.028

Appendix 2a -Effect of timing of urine collection on urinary metabolite profiles - 1 CCB.

Vol.		Urine vol (ml)	Amount excreted in urine (mg) 1-MX	1-MU	17-DMU	17-DMX	AAMU	Enzyme Activities P450IA	NAT	XO
#2	SF Blk	420	11.0	22.9	0.835	3.460	44.54	22.68	0.567	2.07
	2hr	65	2.06	3.71	0.115	0.854	9.034	17.34	0.609	1.80
	4hr	250	3.36	6.38	0.217	1.637	20.87	18.70	0.681	1.89
	6hr	450	2.27	4.55	0.135	1.287	14.05	16.23	0.672	2.00
	8hr	220	1.99	3.70	0.132	0.726	7.341	17.95	0.563	1.85
#9	SO Blk	325	10.0	18.8	0.490	2.392	33.31	26.04	0.534	1.87
	2hr	165	2.90	4.51	0.282	0.554	9.431	30.42	0.559	1.55
	4hr	180	4.21	6.06	0.176	1.018	15.91	25.71	0.607	1.43
	6hr	90	3.34	4.58	0.18	0.98	11.68	20.02	0.595	1.36
	8hr	57	1.51	2.18	0.157	0.406	5.363	22.25	0.592	1.44
#12	JK Blk	190	8.25	19.2	0.376	2.202	36.36	29.01	0.569	2.33
	2hr	200	2.52	6.56	0.096	0.638	16.92	40.78	0.650	2.59
	4hr	225	1.93	4.32	0.137	0.634	14.12	32.13	0.692	2.22
	6hr	195	2.33	6.22	0.064	0.608	13.48	36.24	0.611	2.66
	8hr	51	1.27	3.10	0.062	0.414	7.184	27.92	0.621	2.44
#14	MB Blk	495	44.5	64.1	0.841	8.890	47.47	17.57	0.303	1.44
	2hr	74	8.05	10.9	0.216	1.471	10.28	19.87	0.351	1.35
	4hr	180	9.41	13.1	0.27	1.690	9.003	18.67	0.285	1.39
	6hr	115	5.90	6.62	0.058	1.185	8.351	17.61	0.399	1.12
	8hr	125	5.04	6.40	0.048	1.03	6.995	17.91	0.379	1.26
#16	HH Blk	265	12.9	15.4	2.003	6.601	20.00	7.338	0.413	1.19
	2hr	93	4.52	5.55	0.558	2.189	6.880	7.747	0.405	1.22
	4hr	185	3.08	3.56	0.351	2.105	7.011	6.488	0.513	1.15
	6hr	162	2.75	3.41	0.330	2.246	5.645	5.261	0.477	1.23

	8hr	51	1.99	2.38	0.241	0.854	3.644	9.388	0.454	1.19
#18 SA	Blk	325	22.2	31.1	1.342	9.756	92.17	14.91	0.633	1.40
	2hr	425	8.49	12.7	0.323	5.040	35.15	11.18	0.623	1.49
	4hr	272	4.86	6.45	0.443	3.557	15.98	7.677	0.585	1.32
	6hr	200	2.6	4.23	0.23	2.168	11.36	8.395	0.624	1.63
	8hr	140	3.30	5.28	0.254	1.981	14.20	11.50	0.623	1.59
#19 SH	Blk	12	0.76	1.22	0.020	0.180	2.676	25.79	0.573	1.59
	2hr	130	5.95	9.72	0.288	4.355	22.10	8.676	0.584	1.63
	4hr	155	6.15	9.97	0.421	6.731	20.13	5.387	0.555	1.61
	6hr	125	5.97	9.74	0.275	4.807	18.68	7.155	0.543	1.63
	8hr	100	6.75	9.84	0.271	3.652	27.41	12.05	0.622	1.45

Appendix 2b -Effect of timing of urine collection on urinary metabolite profiles - 4 CCBs.

Vol.		Urine vol (ml)	Amount excreted in urine 1-MX	1-MU	17-DMU (mg)	17-DMX	AAMU	Enzyme Activities P450IA	NAT	XO
#2	SF Blk	450	16.0	27.8	0.949	5.652	52.17	16.99	0.543	1.74
	2hr	200	3.21	6.1	0.292	2.768	12.96	8.049	0.581	1.89
	4hr	760	3.47	7.21	0.562	4.21	20.33	7.368	0.655	2.07
	6hr	505	2.35	4.36	0.505	2.484	9.923	6.699	0.596	1.85
	8hr	168	2.31	4.30	0.283	1.402	6.961	9.680	0.512	1.86
#9	SO Blk	200	14.8	28.6	0.872	3.568	71.01	32.09	0.620	1.93
	2hr	38	3.28	4.69	0.122	0.546	12.28	37.08	0.606	1.42
	4hr	35	2.52	3.78	0.099	0.401	10.00	40.59	0.613	1.49
	6hr	42	2.25	3.53	0.116	0.498	13.58	38.81	0.701	1.56
	8hr	59	2.86	4.63	0.220	1.248	14.20	17.37	0.654	1.61
#12	JK Blk	315	11.4	21.7	0.431	4.951	59.69	18.76	0.642	1.89
	2hr	260	5.50	13.7	0.166	1.679	46.98	39.43	0.709	2.49
	4hr	42	1.84	4.73	0.136	0.523	14.70	40.61	0.691	2.57
	6hr	41	2.39	6.22	0.688	0.9	20.37	32.22	0.702	2.59
	8hr	42	1.72	4.22	0.184	0.553	14.21	36.43	0.704	2.44
#14	MB Blk	345	46.2	55.4	1.221	9.663	27.44	13.36	0.212	1.19
	2hr	75	10.6	13.2	0.404	1.761	10.02	19.26	0.295	1.24
	4hr	80	7.58	9.06	0.422	1.553	6.956	15.19	0.294	1.19
	6hr	70	9.10	10.9	0.268	1.288	5.765	20.04	0.223	1.20
#16	HH Blk	273	6.46	9.03	1.193	4.206	10.43	6.167	0.402	1.39
	2hr	62	2.83	3.14	0.300	0.813	4.545	12.93	0.432	1.11
	4hr	212	2.99	3.58	0.438	2.253	4.594	4.957	0.411	1.19
	6hr	82	1.62	1.94	0.319	1.111	3.999	6.814	0.528	1.19
	8hr	56	2.14	2.58	0.346	1.172	3.275	6.824	0.409	1.20

#18	SA	Blk	170	8.40	11.4	0.314	2.839	24.42	15.61	0.551	1.36
		2hr	430	6.16	9.69	0.313	5.099	20.14	7.059	0.559	1.57
		4hr	450	2.63	4.72	0.171	5.269	9.054	3.116	0.551	1.79
		6hr	170	3.36	4.91	0.272	3.039	11.53	6.521	0.581	1.46
		8hr	102	3.20	5.74	0.298	2.688	17.15	9.711	0.657	1.79
#19	SH	Blk	25	3.07	3.52	0.109	0.898	6.584	14.67	0.499	1.14
		2hr	150	8.06	11.3	0.634	6.847	33.32	7.708	0.631	1.41
		4hr	275	8.88	12.1	0.794	10.60	33.79	5.173	0.616	1.36
		6hr	110	5.74	7.88	0.520	4.651	19.99	7.229	0.594	1.37
		8hr	70	7.06	9.25	0.63	3.663	20.09	9.938	0.551	1.31

Appendix 3 - 0-8 h urinary metabolite profiles: A population study.

Vol	Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#2	SF	29	F	24.42		Y	120	1.852	0.201	4.85	9.71	11.97	0.813
#4	AT	28	F	23.26			85	0.821	0.041	0.90	2.10	10.71	0.048
#7	RB	34	M	22.09			115	1.420	0.194	2.79	5.87	16.40	0.570
#9	SO	24	F	19.30	+	Y	240	3.372	0.343	12.7	9.83	41.40	0.794
#11	JM	30	F	17.40			70	1.204	0.645	3.60	3.67	7.800	0.379
#12	JK	46	F	22.49			205	1.582	0.576	6.69	16.3	55.63	0.444
#13	PL	25	M	17.04			120	3.008	0.471	3.01	2.40	8.856	0.709
#14	MB	32	M	24.66	+		96	1.392	0.133	5.34	8.4	5.833	0.348
#15	MH	33	M	24.49			195	2.154	0.265	4.30	12.7	19.63	0.520
#16	HH	26	F	21.40		Y	120	2.428	0.331	4.5	5.49	7.848	1.034
#17	CP	25	M	22.26	+		50	0.433	0.069	1.88	4.61	4.580	0.207
#18	SA	35	M	23.07			130	2.853	0.240	5.49	8.59	27.84	0.473
#19	SH	50	F	24.60		Y	120	2.109	0.148	3.36	5.10	12.50	0.738
#20	TH	24	M	22.28	+		110	0.730	0.470	2.56	4.64	6.378	0.528
#21	CS	36	F	23.37			59	0.895	0.048	1.30	2.71	6.004	0.499
#22	PB	50	M	23.70			388	3.666	0.209	5.14	10.6	46.75	0.442
#23	MF	35	M	25.50	+		325	2.060	0.123	7.21	12.0	14.75	0.848
#24	DP	25	M	-	-	-	330	1.537	0.115	9.24	16.8	11.59	ND
#25	KM	25	M	-	-	-	365	2.244	0.219	7.53	16.7	14.58	ND
#26	AY	25	M	-	-	-	265	1.303	0.100	13.9	17.5	8.996	ND
#27	SH	27	M	-	-	-	380	2.622	0.266	12.1	36.1	29.98	ND
#28	AY	38	M	-	-	-	500	3.12	0.31	8.16	3.84	26.23	ND
#29	JR	32	M	-	-	-	900	10.22	0.126	14.2	24.6	14.29	ND
#30	SV	26	M	-	-	-	1250	11.62	0.325	18.9	31.5	32.2	ND
#31	MK	29	M	-	-	-	250	0.562	0.387	15.2	24.3	30.07	ND
#32	RR	28	M	-	-	-	305	3.977	0.326	33.4	30.5	29.02	ND
#33	IP	28	M	-	-	-	380	2.318	0.167	9.50	15.2	13.76	ND
#34	LE	26	M	-	-	-	550	3.162	0.357	11.7	20.8	28.74	ND
#35	JS	28	M	-	-	-	1100	14.52	0.253	11.6	28.3	43.56	ND

Appendix 3 - 0-8 h urinary metabolite profiles: A population study.

Vol	Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#36	JW	24	M	–	–	280	2.752	0.067	15.1	19.8	17.24	ND
#37	AE	29	M	–	–	280	0.660	0.316	23.0	42.4	20.58	ND
#38	AM	30	M	–	–	500	5.195	0.28	44.7	104.	57.18	ND
#39	PH	32	M	–	–	530	3.630	0.143	24.1	25.2	26.52	ND
#40	CB	39	F	–	–	470	2.486	0.267	11.2	19.9	21.94	ND
#41	JC	36	F	–	–	260	2.446	0.140	6.90	13.8	8.457	ND
#42	NT	27	M	–	–	800	4.656	0.312	27.9	22.1	19.82	ND
#43	DD	30	M	–	–	420	3.133	0.394	3.16	3.99	9.634	ND
#44	KS	29	F	–	–	650	4.459	0.201	11.5	13.0	33.16	ND
#45	JG	40	M	–	–	625	7.281	0.75	32.6	29.9	32.6	ND
#46	DL	43	F	22.18		780	10.55	1.365	25.3	34.9	20.99	1.099
#47	DP	54	F	28.71	+	Y	930	15.37	1.404	20.4	23.3	23.91	0.920
#48	DK	43	F	21.41	+		500	7.54	1.195	26.3	27.3	42.78	0.225
#49	CG	61	M	26.22			1500	6.99	0.255	12.6	27.5	15	1.815
#50	RG	52	M	24.14			1210	31.25	3.642	35.2	55.3	101.6	5.699
#51	DH	47	M	25.29			1350	37.42	3.766	43.0	56.8	68.21	2.565
#52	DF	50	M	21.49	+		770	13.23	1.763	29.3	41.4	83.41	1.024
#53	PS	34	M	25.69	+		1560	33.83	2.932	72.8	87.0	32.99	5.865
#54	CD	33	F	22.45			1350	20.41	1.903	28.0	20.5	26.77	4.509
#55	VH	55	F	29.26			1050	9.418	0.472	16.9	17.7	11.43	0.189
#56	RD	61	M	25.49			550	13.04	1.435	35.1	30.6	65.80	1.787
#57	AG	39	M	18.62			1390	22.08	1.139	64.6	51.3	173.9	7.506
#58	SS	56	M	34.25			480	7.363	0.652	31.7	22.1	20.32	0.561
#59	DD	57	M	30.47			805	11.52	1.465	12.9	17.3	44.05	2.817
#60	MW	46	M	22.62			735	8.797	0.624	16.5	21.2	27.38	0.330
#61	PB	45	M	22.53			2250	42.18	2.115	41.2	51.5	105.8	2.745
#62	AB	52	M	28.13			1040	11.80	2.225	22.4	19.1	54.18	0.842
#63	CH	28	M	26.48			470	8.272	0.911	23.3	23.9	15.80	0.479
#64	RB	20	M	27.36			1070	22.60	1.423	29.8	28.1	35.35	2.461

Appendix 3 - 0-8 h urinary metabolite profiles: A population study.

Vol		Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#65	AG	26	M	22.46			860	12.04	0.928	28.9	34.9	34.49	0.507
#66	DB	38	F	22.60	+	Y	180	6.951	2.608	30.3	23.0	14.42	0.775
#67	RW	56	M	34.33	+		550	7.419	0.907	21.5	5.04	26.08	0.456
#68	AJ	32	M	26.97	+		1124	18.48	2.911	67.1	67.0	31.11	2.292
#69	MD	36	M	24.10	+		850	10.42	1.377	18.2	15.0	36.20	0.535
#70	RC	58	M	23.39			650	15.49	2.444	16.9	28.9	59.39	3.029
#71	BK	39	M	26.06			1050	18.59	1.89	31.7	40.0	26.13	1.039
#72	MB	32	M	24.93			1380	15.91	1.255	22.7	21.7	27.82	1.794
#73	AA	29	M	29.71			950	28.93	2.517	31.3	50.4	54.43	2.650
#74	SM	30	M	25.62			500	13.6	1.58	17.2	25.0	57.32	0.99
#75	PB	24	M	19.48			330	7.748	1.296	39.9	7.92	107.3	0.412
#76	YK	33	F	20.31			375	4.935	0.57	5.94	6.97	39.89	0.663
#77	SG	41	F	22.00			710	17.28	2.946	30.1	31.8	40.12	1.469
#78	JP	20	F	22.20		Y	475	10.75	1.092	35.6	39.6	27.27	2.09
#79	CA	20	F	20.89		Y	200	4.614	2.12	11.6	26.2	22.1	0.654
#80	CH	32	M	21.77			440	22.63	1.975	28.8	38.2	70.51	2.002
#81	AS	33	M	25.06			690	12.10	0.931	37.2	39.1	35.47	1.580
#82	PB	55	M	19.74			470	4.817	0.225	34.0	8.04	44.48	0.381
#83	WC	51	M	24.38			1020	11.66	1.193	14.4	20.5	53.10	1.785
#84	JY	55	M	26.09			785	8.870	0.949	6.76	11.2	33.68	0.855
#85	RP	62	M	24.80			1275	14.82	1.746	15.4	23.2	55.95	4.156
#86	AA	31	F	23.01		Y	410	5.428	1.467	5.76	7.02	19.67	1.738
#87	JC	34	F	22.12			1260	14.16	1.738	19.3	24.7	22.18	2.482
#88	WE	49	F	29.55			480	2.587	0.393	8.16	6.19	13.99	0.787
#89	JG	57	F	20.72		Y	525	6.536	0.934	8.4	11.3	51.69	1.795
#90	YC	38	F	21.52			185	6.630	1.250	25.2	30.0	40.92	0.762
#91	HB	38	F	21.10			400	3.376	0.468	5.86	6.18	13.18	0.052
#92	JH	25	F	24.80	+		470	6.222	1.015	13.3	19.1	54.52	0.869
#93	GW	33	F	22.49	+		1005	22.83	2.281	89.1	79.5	109.9	4.803

Appendix 3 - 0-8 h urinary metabolite profiles: A population study.

Vol		Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#94	MR	32	M	23.95			585	11.57	1.000	33.0	35.4	41.58	1.585
#95	MB	32	M	21.57			660	11.96	1.590	76.4	25.5	78.17	0.976
#96	SC	27	F	19.62	+	Y	630	9.859	1.045	13.5	17.7	19.20	1.650
#97	PA	48	F	28.02			800	15.98	2.04	26.6	27.3	43.08	4.064
#98	SH	23	F	19.83	+	Y	750	7.59	1.342	10.6	9.65	22.49	1.275
#99	AP	21	F	22.54			600	4.692	0.708	13.0	17.3	19.29	0.324
#100	NC	29	F	20.83	+		820	10.78	1.336	52.2	36.7	51.55	1.344
#101	HC	26	M	27.83			330	10.15	1.884	19.7	26.2	31.40	1.560
#102	TW	40	M	29.26			1350	16.86	1.363	19.5	36.0	14.66	0.607
#103	CL	57	M	24.72			860	25.93	2.107	28.6	39.2	96.39	3.534
#104	JP	58	M	23.17			1075	27.65	2.762	50.3	64.7	82.90	3.268
#105	AW	38	M	25.77	+		870	11.97	1.218	20.5	31.5	19.00	1.461
#106	WP	43	M	23.05			580	1.722	0.348	12.8	10.8	25.20	0.603
#107	SC	32	F	21.82		Y	185	4.649	1.800	7.98	14.3	42.97	1.320
#108	ER	49	F	20.09			1360	7.316	0.163	3.07	8.98	10.44	2.271
#109	MH	26	M	24.31			850	12.75	1.19	12.7	16.0	30.72	2.397
#110	JS	30	M	24.93			970	27.18	2.386	32.6	63.0	146.4	2.628
#111	AF	39	M	28.08			235	3.823	1.621	6.63	10.7	47.84	0.589
#112	RB	41	M	25.77			510	12.28	1.555	20.1	41.3	113.9	1.744
#113	IB	53	M	27.32			685	13.50	2.000	10.4	24.8	13.04	2.363
#114	OC	57	M	26.09			375	8.73	1.646	10.2	17.4	63.53	2.531
#115	FW	60	M	22.53	+		1000	16.05	1.62	15.9	33.7	99.68	1.92
#116	LQ	23	M	22.91	+		405	6.253	1.036	12.6	22.1	66.74	0.441
#117	GS	62	M	23.35	+		755	13.03	0.747	26.5	46.0	33.26	0.649
#118	KW	29	M	21.82			1380	18.20	0.828	22.7	75.5	37.99	2.373
#119	JB	59	M	26.92			275	8.175	1.240	9.55	17.6	63.50	1.012
#120	DM	51	M	27.99			620	17.40	1.760	12.0	22.1	89.47	4.098
#121	MC	47	M	24.82			370	1.302	0.266	10.0	13.3	21.03	0.188
#122	BT	44	M	20.26			440	8.549	1.064	15.6	32.5	12.36	3.586

Appendix 3 - 0-8 h urinary metabolite profiles: A population study.

Vol		Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#123	HL	40	M	24.76			450	4.653	1.197	24.5	47.3	70.17	2.137
#124	GB	35	M	23.87			530	3.943	0.492	9.10	14.7	48.56	0.540
#125	JA	24	M	23.86			700	10.32	1.512	9.65	9.77	36.58	2.086
#126	VB	56	F	26.13			355	7.188	1.309	12.5	12.8	18.59	1.522
#127	MB	52	F	24.10			830	8.416	0.514	24.3	53.4	30.27	2.365
#128	MS	36	F	27.42		Y	410	10.48	1.439	15.4	17.4	32.89	2.537
#129	ML	47	F	38.01	+		520	9.172	0.759	31.4	78.4	28.63	1.435
#130	DB	53	M	25.12			1080	5.389	1.490	13.4	13.3	60.30	1.285
#131	FM	56	M	25.85			740	10.01	0.273	19.4	41.7	27.41	1.990
#132	SB	30	M	23.95	+		2055	4.623	0.965	26.9	25.1	44.49	4.171
#133	SK	37	F	21.52	+		930	5.886	0.344	8.36	10.7	72.39	1.664

Appendix 4 - 2-6 h urinary metabolite profiles: A population study.

Vol	Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#134	AD	18	F	22.57	+	Y	200	0.806	0.272	6.09	7.46	18.9	0.236
#135	EC	49	F	23.07			90	0.548	0.146	7.98	6.80	15.2	0.134
#136	ST	42	F	25.99			100	0.709	0.267	3.14	3.99	4.92	0.476
#137	EP	41	F	26.09		Y	220	1.249	0.211	7.09	4.49	7.52	0.371
#138	KW	18	F	20.85		Y	110	0.509	0.042	1.06	1.33	1.72	0.139
#139	DM	24	F	20.70			200	2.116	0.42	14.2	7.74	36.2	0.754
#140	AM	32	F	24.93			180	0.961	0.151	2.03	2.80	4.18	0.208
#141	VH	57	F	25.98			180	0.932	0.102	3.96	4.82	7.53	0.169
#142	RW	45	F	27.85			380	4.145	0.399	6.06	11.6	10.3	0.209
#143	LH	20	F	26.95			245	0.644	0.080	1.50	1.67	6.12	0.034
#144	LP	21	F	20.75	+	Y	125	1.468	0.401	3.16	4.85	10.8	0.256
#145	TH	17	F	21.63	+		120	0.326	0.03	0.92	1.17	3.97	0.034
#146	JN	22	F	22.67	+	Y	8	0.096	0.021	0.29	0.32	1.14	0.007
#147	JW	25	F	22.00		Y	150	1.278	0.226	3.70	2.68	2.57	0.297
#148	JW	22	F	21.07	+		300	1.335	0.339	5.16	4.89	12.7	0.225
#149	MW	46	F	23.78			90	0.823	0.077	2.26	2.65	6.09	0.044
#150	CC	23	F	28.58		Y	120	0.536	0.154	0.92	0.86	7.13	0.032
#151	JD	50	F	28.00			100	1.113	0.089	1.21	0.85	3.60	0.071
#152	DE	47	F	22.17			80	0.6	0.067	2.37	3.45	4.98	0.075
#153	JWW	38	F	21.25		Y	100	0.391	0.105	1.44	5.73	6.44	0.074
#154	JH	22	M	17.45			140	1.26	0.103	2.04	3.54	3.56	0.123
#155	PA	37	F	21.14			60	0.277	0.052	0.84	0.74	2.00	0.010
#156	PL	22	M	22.06	+		50	0.359	0.078	0.63	2.15	2.79	0.055
#157	EW	25	F	25.52	+	Y	180	0.594	0.100	3.54	1.48	10.0	0.064
#158	JH	26	F	20.56			70	0.833	0.078	1.17	4.16	1.36	0.077
#159	LT	20	F	20.09	+	Y	70	0.569	0.052	0.57	1.49	3.05	0.491
#160	LW	21	F	23.17			80	1.044	0.14	1.28	4.07	4.98	0.065
#161	SP	26	F	19.64			100	2.652	0.376	2.06	8.86	8.95	0.234
#162	CD	32	F	20.90		Y	110	1.043	0.206	2.41	2.19	5.18	0.244

Appendix 4 - 2-6 h urinary metabolite profiles: A population study.

Vol	Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#163	TM	25	F	22.58		Y	70	0.686	0.058	1.46	6.82	5.54	0.184
#164	AB	80	M	24.66			300	3.291	0.372	3.33	23.1	13.2	ND
#165	ST	20	F	21.48	+	Y	50	0.331	0.035	1.59	3.93	4.21	0.046
#166	SB	24	M	21.57			50	0.396	0.025	1.70	1.47	2.55	0.064
#167	EL	38	F	52.14			150	1.065	0.127	2.44	4.8	11.8	0.309
#168	JD	47	F	23.79	+	Y	90	1.096	0.075	1.14	2.48	5.53	0.094
#169	LH	34	F	22.18			100	1.125	0.084	3.37	3.12	9.13	0.123
#170	PD	42	M	25.62			110	1.377	0.145	1.67	4.81	15.4	0.226
#171	SaS	25	F	20.27	+	Y	50	0.429	0.022	0.58	0.84	3.04	0.029
#172	SuS	49	F	20.07	+		200	1.578	0.126	3.89	8.08	13.2	0.64
#173	JB	50	F	32.88			200	2.408	0.244	7.31	11.8	28.5	0.56
#174	DF	48	M	24.12			200	2.564	0.192	5.16	10.0	12.1	0.818
#175	DB	31	F	20.20		Y	250	1.382	0.16	1.56	4.03	13.7	0.18
#176	GR	31	M	20.42	+		125	1.131	0.067	2.82	4.73	10.5	0.21
#177	JB	23	F	21.05		Y	50	0.380	0.191	0.59	1.49	1.66	0.275
#178	JB		M		+		110	0.149	0.010	0.78	1.56	8.36	0.035
#179	JK	31	F	20.44	+	Y	80	2.424	0.032	1.30	2.32	1.61	0.404
#180	AS	33	F	23.80			150	1.231	0.111	2.15	3.83	3.65	0.127
#181	EB	54	F	21.09	+		70	0.461	0.028	2.09	3.93	0.85	0.035
#182	EM	23	F	21.81			100	0.831	0.027	0.63	1.03	1.78	0.037
#183	VB	24	F	22.57			90	0.948	0.013	1.75	2.79	3.29	0.162
#184	JK	20	M	25.05			178	0.936	0.167	5.22	7.13	15.1	0.300
#185	AH	24	M	23.19	+		75	1.629	0.007	3.71	8.28	6.34	0.203
#186	AD	23	M	23.37			130	2.236	0.300	8.29	16.4	13.1	1.024
#187	TC	33	M	24.42			205	2.843	0.014	6.21	6.80	18.9	0.276
#188	TA	25	M	23.62			165	0.638	0.095	5.38	10.9	5.86	0.166
#189	RL	27	M	22.29			52	0.931	0.017	1.20	1.28	2.98	0.244
#190	MD	27	M	26.39	+		160	1.345	0.161	4.12	4.33	2.88	0.054
#191	TL	31	M	23.27			70	0.933	0.010	4.73	1.03	4.50	0.379

Appendix 4 - 2-6 h urinary metabolite profiles: A population study.

Vol	Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#192	MR	40	M	29.02	+	73	0.727	0.008	0.95	1.38	1.72	0.094
#193	EJ	43	M	27.86		95	0.717	0.062	1.07	1.54	3.01	0.086
#194	SM	23	F	31.84		140	2.069	0.123	7.17	6.77	9.45	0.337
#195	CP	31	F	21.52	+	90	0.446	0.114	2.20	2.97	2.88	0.207
#196	MP	19	F	23.90		Y 70	0.321	0.056	0.89	1.51	1.82	0.040
#197	SB	59	F	26.29		30	0.310	0.007	0.47	0.74	1.95	0.044
#198	JS	42	F	23.83	+	75	0.051	0.008	0.51	0.30	5.22	0.036
#199	JB	58	F	31.01		60	0.798	0.121	1.62	3.05	5.89	0.171
#200	NR	43	F	21.05		130	0.904	0.059	1.68	2.22	1.56	0.105
#201	LB	34	F	24.10		115	0.642	0.020	1.38	1.50	1.12	0.212
#202	JM	47	F	22.67		70	0.672	0.136	0.87	1.29	2.72	0.250
#203	MG	59	F	25.58		50	0.769	0.159	0.84	1.35	2.44	0.231
#204	VL	44	F	24.49		240	1.015	0.055	2.12	2.09	2.39	0.129
#205	LH	21	F	19.98		55	0.326	0.064	1.23	1.94	1.03	0.086
#206	EP	30	M	28.78		150	1.465	0.307	4.36	3.97	8.70	0.678
#207	GJ	22	M	26.05	+	155	1.286	0.158	3.66	4.74	9.17	0.410
#208	SO	21	M	24.93	+	82	1.628	0.131	2.14	2.83	3.08	0.218
#209	AP	21	F	23.69	+	Y 270	3.804	0.164	0.30	0.34	3.71	1.053
#210	JP	47	F	22.17	+	23	0.288	0.017	1.13	1.26	2.03	0.007
#211	RP	47	M	24.12	+	372	1.525	0.167	6.99	6.75	4.53	0.316
#212	GP	25	M	20.87		245	1.771	0.058	6.00	7.83	1.33	0.247
#213	CR	23	F	23.39	+	Y 175	1.179	0.134	2.19	2.99	9.02	0.234
#214	WO	53	M	27.59		230	3.585	0.418	12.8	21.1	28.6	1.258
#215	GO	50	F	22.91		96	0.302	0.030	1.49	1.93	1.75	0.053
#216	RW	48	F	31.51		80	1.124	0.372	6	4.75	6.68	0.452
#217	BD	48	F	23.83		75	0.301	0.016	0.41	0.59	1.50	0.013
#218	HM	18	F	22.28		135	0.789	0.021	2.08	1.72	6.99	0.062
#219	SM	20	F	20.91		Y 155	0.463	0.062	1.00	1.36	3.86	0.063
#220	LF	34	F	23.93		160	1.481	0.124	2.35	2.36	9.55	0.195

Appendix 4 - 2-6 h urinary metabolite profiles: A population study.

Vol		Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#221	BM	55	F	23.59		Y	130	2.967	0.198	2.55	2.32	10.0	0.848
#222	MH	52	F	21.37			150	1.252	0.109	2.57	3.93	4.91	0.048
#223	MT	47	F	22.00			100	0.639	0.024	0.78	1.20	1.75	0.34
#224	BD	56	F	22.92			55	0.400	0.033	0.99	1.60	1.25	0.090
#225	HH	58	F	24.84		Y	60	0.168	0.018	0.46	0.25	0.45	0.073
#226	MP	51	F	29.09			125	1.935	0.093	3.16	5.42	4.22	0.508
#227	JW	28	F	17.52			50	0.255	0.016	0.52	1.11	0.28	0.057
#228	JC	56	F	27.30			90	0.685	0.074	0.91	1.12	5.94	0.149
#229	NB	22	M	22.82			300	2.031	0.081	1.30	1.63	4.34	0.156
#230	MB	29	M	23.81			130	0.419	0.013	1.63	2.82	1.66	0.057
#231	AH	31	M	24.12			120	0.96	0.114	11.6	0.51	1.88	0.217
#232	CB	32	M	23.59			50	0.1	0.009	0.37	0.63	2.88	0.017
#233	RB	35	M	19.91	+		45	0.216	0.038	0.52	0.52	5.69	0.205
#234	DR	43	M	26.52			150	1.453	0.175	0.85	1.14	15.4	0.273
#235	BA	45	M	26.52			50	0.546	0.074	1.56	2.37	1.33	0.066
#236	MH	47	M	24.54			75	0.366	0.049	3.01	4.54	0.62	0.183
#237	RH	49	M	22.29			150	0.573	0.028	3.62	4.19	6.30	0.253
#238	AS	25	M	21.49			275	0.995	0.093	4.74	4.40	10.4	0.302
#239	GT	31	M	23.58	+		150	1.561	0.172	13.0	14.1	18.2	0.223
#240	SR	32	M	24.66			120	1.075	0.116	5.7	5.18	15.2	0.260
#241	DC	33	M	22.78			190	2.171	0.203	12.7	25.8	45.0	1.077
#242	SL	35	M	27.52			20	0.124	0.037	0.89	1.27	1.36	0.059
#243	SK	37	M	26.52	+		240	0.885	0.105	2.17	3.41	4.41	0.048
#244	RH	37	M	24.37	+		190	4.592	0.144	4.35	5.06	4.61	0.655
#245	RC	39	M	25.50			10	0.119	0.005	0.16	0.35	0.88	0.019
#246	RT	48	M	25.06			100	0.696	0.139	2.21	2.69	6.27	0.21
#247	PW	53	M	17.57	+		20	0.181	0.014	0.72	1.81	1.74	0.017
#248	LM	69	M	26.06	+		50	0.299	0.018	7.93	0.15	1.11	0.037
#249	DC	36	M	20.98			300	0.816	0.006	1.32	2.71	2.52	0.159

Appendix 4 - 2-6 h urinary metabolite profiles: A population study.

Vol	Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#250	AW	56	M	22.72	+		75	0.205	0.012	0.67	1.04	1.13	0.166
#251	JD	52	F	21.01		Y	240	1.176	0.139	2.41	5.50	7.31	0.458
#252	JH	54	M	20.26	+		60	0.414	0.012	0.52	2.20	1.90	0.053
#253	IM	66	M	20.91			110	1.246	0.056	3.51	8.67	4.74	0.202
#254	GT	83	F	22.91			175	1.568	0.077	6.24	4.01	5.50	0.42
#255	RT	90	M	31.16			60	0.325	0.039	2.90	1.76	2.72	0.041
#256	VG	76	F	23.13			280	0.938	0.056	0.98	2.62	3.26	0.364
#257	RG	77	M	29.87			225	2.504	0.042	1.32	5.05	1.71	0.461
#258	JJ	69	M	28.55			40	0.653	0.036	2.56	3.16	2.45	0.092
#259	IJ	70	F	17.90			90	0.607	0.052	0.30	1.15	0.33	0.211
#260	LP	76	M	23.95			200	1.48	0.226	7.88	1.43	3.32	1.082
#261	WP	75	F	25.46	+		190	1.922	0.127	3.46	8.81	5.84	0.482
#262	EF	75	F	22.50			90	0.507	0.040	1.00	2.47	2.39	0.063
#263	HF	78	M	25.35			100	1.656	0.076	3.33	10.4	5.67	0.218
#264	EC	90	F	22.80			80	0.853	0.045	1.29	3.77	3.00	0.250
#265	DS	88	F	28.99			50	0.546	0.044	2.67	2.80	1.71	0.123
#266	AR	71	M	23.03			80	2.537	0.161	5.24	20.3	4.36	0.547
#267	EN	86	F	26.66			40	0.319	0.033	0.98	3.03	0.77	0.144
#268	LA	65	F	20.70			150	0.514	0.016	1.00	2.36	1.60	0.109
#269	WB	75	M	24.34			50	0.385	0.062	2.04	3.55	2.92	0.215
#270	GW	81	F	22.07			15	0.210	0.006	0.29	0.85	0.33	0.043
#271	ER	74	M	22.43	+		80	0.714	0.065	2.29	7.38	3.56	0.144
#272	BR	68	F	22.20			50	0.568	0.106	2.66	8.13	3.9	0.192
#273	GC	59	F	26.47	+		90	0.936	0.210	4.69	11.5	1.88	0.258
#274	TR	50	F	25.10		Y	95	1.493	0.136	1.51	4.25	5.25	0.249
#275	VC	61	F	22.63			65	0.801	0.118	2.53	5.45	2.10	0.243
#276	JP	44	F	23.59	+	Y	100	1.312	0.263	4.95	10.3	7.03	0.289
#277	SF	41	F	30.22	+	Y	196	1.791	0.213	5.51	9.70	11.2	0.343
#278	PT	56	F	21.64			90	1.009	0.032	1.50	3.07	0.83	0.314

Appendix 4 - 2-6 h urinary metabolite profiles: A population study.

Vol	Age	Sex	BMI	Smoker	OCS	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#279	MD	65	F	30.42	+	70	0.842	0.136	0.82	8.50	2.68	0.157
#280	PH	44	F	25.49	+	275	1.641	0.057	9.38	8.35	5.02	0.297
#281	DH	49	M	26.79	+	230	2.035	0.087	6.89	16.0	4.45	0.126
#282	MM	28	M	19.11	+	280	2.875	0.106	5.27	7.31	2.07	2.060
#283	CM	23	M	24.80	+	720	0.799	0.158	2.81	3.67	3.16	0.208

Appendix 5 -Urinary caffeine metabolites in patients with carcinoma of the lung, head and neck.

Vol		Age	Sex	BMI	Smoker	Cancer Type		Medication	Urine vol (ml)	Amount excreted in urine (mg)					
										17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#28	DH	63	M	22.6	+	S	B	Primadone	150	0.741	0.033	2.41	1.65	7.52	0.12
#29	AM	70	M	27.2	+	A	B	Temazepam	40	0.410	0.087	0.93	1.97	4.25	0.097
#30	GP	79	M	20.2		S	H	Carbamazepine	70	0.231	0.021	1.26	2.60	2.15	0.079
#31	HH	71	M	20.1	+	O	B	Heroin	45	0.372	0.046	4.43	4.25	3.06	0.101
#32	IT	65	M	25.7	+	O	B		10	0.077	0.008	1.14	0.37	0.49	0.006
#33	PE	47	M	25.1		A	H	Temazepam	60	1.254	0.057	5.96	5.85	6.85	0.273
#34	WG	76	M	22.0		O	B	GTN	70	0.404	0.063	2.11	3.42	2.73	0.102
#35	IB	76	M	26.9		S	H		110	1.356	0.069	9.18	1.11	4.35	0.713
#36	SI	82	F	20.7		S	B		120	0.186	0.043	1.50	0.94	2.19	0.163
#37	AD	69	M	23.3	+		B		100	1.847	0.104	3.92	10.4	3.67	0.465
#38	TH	77	M	22.2	+		B		125	0.343	0.091	0.92	11.1	4.89	0.113
#39	JB	78	M	31.8				Warfarin	155	1.774	0.120	1.37	5.59	2.13	0.486
#40	MT	71	F	19.7			B		55	0.482	0.067	0.92	0.70	1.69	0.121
#41	RK	79	M	25.4		S	B		100	0.813	0.178	0.67	2.84	4.10	0.176
#42	RH	74	M	28.0		S	B		50	0.389	0.012	2.40	2.79	2.22	0.019
#43	DR	74	M	28.5			B		140	3.960	0.294	4.49	12.4	5.62	1.121
#44	AH	79	M	27.9			B	Nifedipine	10	0.108	0.009	0.16	0.30	0.38	0.016
#45	DJo	49	M	21.0	+	S	B		110	0.884	0.046	1.42	5.54	1.43	0.092
#46	HS	79	M	26.3	+	S	B	Frumil	90	0.558	0.046	2.12	8.15	4.50	0.041
#47	JG	66	M	23.9		S	B		180	1.323	0.025	1.16	5.17	1.56	0.176
#48	MA	48	M	17.3	+	S	B	Ventolin	110	1.006	0.106	4.74	6.76	6.61	0.104
#49	DHu	63	F	24.0		O	B		55	0.337	0.015	0.56	2.04	2.41	0.022
#50	RP	62	M	25.2	+	S	B		100	0.843	0.069	0.52	2.40	3.31	0.15
#51	GJ	82	M	23.3		S	B	Lactulose	75	1.92	0.091	2.12	3.85	8.56	0.251

S Squamous, O Oat, SW Swan, B Bronchi, H Head or neck.

Appendix 5 -Urinary caffeine metabolites in patients with carcinoma of the lung, head and neck.

Vol		Age	Sex	BMI	Smoker	Cancer Type		Medication	Urine vol (ml)	Amount excreted in urine (mg)					
										17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#1	PW	70	M	22.8	+	S	B	Coproximol	130	0.863	0.106	2.70	5.01	6.94	0.273
#2	LF	59	M	23.5	+	O	B		90	0.723	0.048	1.50	5.15	3.93	0.125
#3	JG	52	M	22.8		S	B	Frumil	125	0.641	0.118	1.59	2.45	4.07	0.241
#4	AP	66	M	20.9		S	B	Penicillamine	40	0.335	0.085	2.29	4.50	3.09	0.324
#5	HF	74	M	20.6		S	B		50	0.385	0.073	1.44	2.57	4.72	0.127
#6	NS	63	M	26.0		O	B	Regulan	120	0.979	0.099	3.66	6.02	2.51	0.187
#7	TT	63	M	21.3	+	O	B	Temazepam	90	0.368	0.122	2.00	6.60	7.07	0.222
#8	MD	47	F	18.3		S	B	Metoprolol	30	0.116	0.040	0.37	1.40	0.88	0.082
#9	ECh	66	F	18.5		O	B		15	0.247	0.086	1.41	3.34	1.83	0.099
#10	ED	79	F	17.3		S	H	Lactulose	150	0.622	0.12	2.27	2.73	6.48	0.445
#11	JS	56	F	35.8			H	Naproxin	120	1.278	0.192	2.43	3.34	5.16	0.514
#12	PS	54	M	23.5		S	H	Temazepam	180	1.216	0.049	3.15	1.81	5.17	0.444
#13	ECo	83	M	19.1			B	Lactulose	50	0.294	0.032	0.52	1.31	1.86	0.074
#14	FG	64	F	20.3		O	B	Codanthrusate	50	0.477	0.048	1.69	3.15	2.46	0.152
#15	BB	62	M	22.2	+	S	B		100	0.544	0.027	1.08	2.73	6.18	0.045
#16	EK	81	M	21.7		S	B	Codeine	40	0.560	0.026	0.44	1.34	1.35	0.124
#17	EP	69	F	27.6		S	B		395	2.524	0.272	6.04	14.6	13.5	0.837
#18	LA	66	M	20.7		S	H	Paracetamol	60	0.180	0.052	1.28	3.07	4.83	0.035
#19	LE	66	M	25.0		S	H		15	0.119	0.029	0.64	0.78	0.82	0.031
#20	PR	78	M	23.9		SW	B	Paracetamol	260	2.438	0.140	3.57	5.60	8.22	1.149
#21	PH	47	M	23.1		S	H		100	0.592	0.126	2.00	3.98	3.78	0.438
#22	JH	66	M	21.7		S	H		150	1.042	0.12	6.20	1.88	6.08	0.340
#23	IS	73	F	25.4		S	B		150	1.149	0.093	2.09	4.45	17.4	0.259
#24	PD	78	M	24.9		O	B	Ventolin	50	0.200	0.018	1.96	2.25	1.19	0.05
#25	CP	67	M	31.9	+	S	B		75	0.386	0.026	0.73	0.76	1.95	0.037
#26	LB	66	F	23.8		S	B	Oxazepam	5	0.022	0.013	0.42	0.46	0.54	0.013
#27	WW	70	M	18.6	+	S	B	Dexamethasone	210	1.871	0.048	3.38	5.69	3.43	0.273

S Squamous, O Oat, SW Swan, B Bronchi, H Head or neck.

Appendix 6 -Urinary caffeine metabolites in patients with leukaemia .

Vol		Age	Sex	BMI	Smoker	Cancer Type	Medication	Urine vol (ml)	Amount excreted in urine (mg)					
									17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
1	TT	51	F	21.1		L	Ventolin	405	1.429	0.190	6.21	7.49	3.75	0.846
2	ML	74	F	31.7		CLL		70	0.435	0.017	0.62	1.47	1.26	0.033
3	LB	69	M	27.1		CLL	Warfarin	55	0.541	0.152	3.57	6.70	3.67	0.134
4	JH	71	F	26.3		CLL		80	0.472	0.082	2.67	6.28	5.58	0.093
5	MU	47	F	21.4		AML		400	1.14	0.08	1.68	3.08	1.48	0.276
6	DH	24	M	23.4	+	HD		175	1.137	0.077	3.07	5.38	2.58	0.096
7	JS	53	M	25.9		NH		180	1.850	0.221	2.55	3.77	1.73	0.336
8	HW	86	F	17.3		CML	Frumil	100	0.175	0.014	1.37	2.28	1.98	0.234
9	WN	78	M	24.5		M		100	0.346	0.018	0.70	2.78	1.99	0.11
10	GM	65	M	24.8		CML	Fluconazole	375	4.14	0.172	5.70	10.1	35.8	1.545
11	MJ	76	F	17.9		CLL		90	0.300	0.048	1.08	3.92	6.69	0.102
12	GB	59	M	23.3		CLL		120	1.017	0.043	1.44	2.16	8.66	0.218
13	RH	43	M	28.5	+	HD	Prothiaden	70	1.563	0.088	2.86	6.16	11.8	0.277
14	AP	32	F	22.5		HD		85	0.737	0.058	1.99	1.95	6.68	0.160
15	BE	62	F	26.8		CLL	Codeine	60	3.485	0.026	1.78	2.91	1.97	0.052
16	JP	28	F	19.8		HD		25	0.062	0.007	0.16	0.37	1.08	0.029
17	MS	69	F	20.3		M	Piroxicam	110	0.386	0.028	0.95	2.22	4.96	0.082
18	HW	51	F	20.5		CLL		100	0.876	0.104	3.96	5.33	7.69	0.264
19	IW	86	F	21.4		CLL	Glibenclamide	80	0.455	0.035	1.90	7.56	2.20	0.071
20	VN	59	F	28.3		ALL	Thyroxine	240	0.542	0.045	0.78	1.23	2.10	0.103
21	MM	81	F	26.4	+	M		180	1.009	0.057	1.20	5.25	4.33	0.397
22	JW	71	M	21.8		M		250	0.38	0.005	1.37	0.31	0.66	0.017
23	SP	68	M	24.6		HAIRY	Interferon	60	0.813	0.055	1.61	2.55	2.27	0.175
24	EHa	32	F	24.8		AML	Acyclovir	175	1.221	0.068	6.43	11.0	2.84	0.206
25	ECh	70	F	19.8		AML	Acyclovir	100	0.625	0.023	13.3	5.48	1.37	0.303
26	MD		F	18.0	+	CLL		90	0.400	0.046	2.21	6.32	4.77	0.046

M Myeloma, L Lymphoma, ALL Acute Lymphocytic Leuk, CML Chronic Myeloid Leuk, HD Hodgkin's, NH Non-Hodgkin's Lymphoma

Appendix 6 -Urinary caffeine metabolites in patients with leukaemia .

Vol		Age	Sex	BMI	Smoker	Cancer Type	Medication	Urine vol (ml)	Amount excreted in urine (mg)					
									17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
27	JS	61	F	30.3		CLL	Frumil	75	0.711	0.078	2.20	3.41	3.54	0.155
28	PB	48	F	22.0		AML	Thyroxine	75	0.246	0.022	0.86	1.82	3.63	0.036
29	RB	70	M	24.1		CLL	Naproxin	60	0.408	0.017	0.97	1.75	3.34	0.112
30	MC	58	F	34.8		CLL	Paracetamol	90	0.532	0.062	12.3	4.20	8.53	0.207
31	IWa	70	F	29.8		NH	Prothiaden	50	0.737	0.065	2.45	7.18	7.68	0.069
32	LP	73	M	20.5		CLL	Propranolol	150	0.685	0.088	1.21	3.11	6.44	0.183
33	EHu	71	F	25.0		CGL		120	1.173	0.070	1.78	5.62	9.37	0.111
34	WT	80	F	19.2		NH		45	0.345	0.014	0.23	0.91	2.64	0.152
35	HB	80	M	22.8		CLL		80	0.413	0.031	1.59	5.49	7.52	0.099
36	SS	30	M	20.5		ALL		50	0.309	0.022	0.67	1.30	1.62	0.025
37	AG	61	M	22.8	+	CLL	Interferon	275	1.523	0.079	1.38	3.99	2.57	0.825
38	HD	64	F	23.8		CLL		150	1.177	0.091	4.42	1.44	10.0	0.234
39	AP	80	F	25.6		CML	Paracetamol	70	0.719	0.025	0.72	2.18	1.52	0.159
40	DL	71	F	24.8		CLL		470	0.390	0.020	0.76	1.11	2.00	0.178
41	YV	53	F	23.7	+	CLL	Atenolol	325	1.462	0.185	14.6	19.6	8.75	0.581
42	JB	39	M	32.1		CGL		50	0.508	0.026	6.27	2.21	1.81	0.261
43	GM	78	M	18.6		CLL	Prednisolone	60	0.072	0.013	0.13	0.43	0.51	0.006
44	JD	56	M	24.3		NH		275	5.403	0.123	2.91	9.69	4.37	4.094
45	JP	63	F	23.9		CLL		145	2.886	0.068	1.34	3.85	5.51	0.527
46	GC	56	M	26.9		HAIRY		110	2.214	0.113	3.85	6.15	3.36	0.55
47	BC	63	F	23.5		AML		160	1.664	0.075	3.30	4.64	2.55	0.236
48	EHe	77	M	20.4		CGL	Digoxin	7	0.057	0.010	0.06	0.59	0.29	0.014
49	GM	61	M	25.3		HAIRY		50	0.172	0.035	1.25	0.50	0.37	0.026
50	DN	56	F	26.0		CLL	lorazepam	100	0.714	0.034	1.10	3.88	3.91	0.97
51	RL	77	M	23.7		CLL		50	0.163	0.015	0.66	1.53	0.95	0.021
52	KT	43	M	23.3	+	AML		50	0.749	0.068	1.91	3.32	2.33	0.112
53	FL	86	F	25.1		CLL	Aspirin	220	2.378	0.033	1.10	4.38	3.38	0.44
54	SS	60	F	24.5		L		40	0.540	0.032	0.98	0.21	1.16	0.082

M Myeloma, L Lymphoma, ALL Acute Lymphocytic Leuk, CML Chronic Myeloid Leuk, HD Hodgkin's, NH Non-Hodgkin's Lymphoma

Appendix 7 -Urinary caffeine metabolites in patients with colorectal carcinoma.

Vol	Age	Sex	BMI	Cancer Type	Medication	Urine vol (ml)	Amount excreted in urine (mg)					
							17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#1	AH 54	F	21.4	A C	Imodium	150	0.910	0.019	2.23	2.452	1.77	0.2355
#2	RT 73	M	18.9	A R	Codeine	20	0.205	0.02	0.45	1.336	1.40	0.0554
#3	AR 71	M	14.8	C		75	0.497	0.044	2.15	3.906	2.61	0.0945
#4	RW 56	M	22.5	A R	Ventolin	170	1.890	0.255	3.06	4.260	1.65	0.4658
#5	HT 63	F	29.8	A C		125	1.995	0.07	2.88	5.083	3.40	0.4062
#6	MF 77	F	22.8	A R	Nitrazepam	50	0.112	0.031	0.92	1.935	1.49	0.0475
#7	DP 76	M	24.5	S A		20	0.065	0.023	0.42	0.701	0.44	0.0204
#8	VP 76	F	25.4	R		40	1.337	0.037	1.24	3.450	1.39	0.1776
#9	RW 63	F	20.7	C	GTN,Aspirin	30	0.183	0.011	0.72	0.790	0.97	0.0207
#10	VP 79	M	24.1	A R	Digoxin	30	0.794	0.064	0.61	2.701	1.78	0.1986
#11	RM 66	F	20.7	A R		170	1.434	0.066	1.54	3.935	7.77	0.3706
#12	PL 86	F	16.3	A CA		60	0.833	0.034	1.05	4.064	1.89	0.0708

A adeno, S squamous, R Rectum, C Colon, A Anus, CA Caecum.

Appendix 8a -Urinary caffeine metabolites in patients with FAP.

Vol		Age	Sex	Smoker	Urine vol (ml)	17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#1	AM	58	F		750	10.95	1.042	18.50	42.57	69.06	3.66
#2	KB	19	F		800	9.08	1.144	26.45	68.23	168.1	1.888
#3	DB	36	M		700	18.27	0.861	62.51	92.96	39.78	2.268
#4	GG	61	M		210	2.442	0.231	26.48	68.63	25.94	19.75
#5	PR	19	M		400	9.004	0.816	23.31	53.70	55.29	0.268
#6	TG	37	M	+	350	7.679	0.885	36.62	89.38	26.47	0.199
#7	JM	33	F		250	8.155	0.63	23.88	34.72	28.76	0.615
#8	SB	23	M		250	4.495	0.722	19.92	50.49	44.72	0.582
#9	LA	19	F		210	1.862	0.371	15.77	27.55	13.25	0.422
#10	SB	29	F		450	13.75	0.418	26.17	34.91	22.38	3.114
#11	DR	52	M		600	19.23	0.798	19.93	55.72	24.91	1.416
#12	JB	20	M		260	4.295	0.639	22.09	29.93	19.07	0.696
#13	RF	45	M	+	240	5.181	0.638	30.88	76.24	66.31	0.319
#14	AM	27	F	+	625	7.687	0.268	33.15	66.03	19.15	0.468
#15	RT	41	M		575	9.2	0.638	8.630	16.45	22.03	1.817
#16	JK	56	F		800	13.44	0.768	15.86	39.07	11.22	1.568
#17	MB	41	M		200	3.356	0.68	55.69	123.0	41.41	0.264
#18	LM	21	F		275	7.108	1.009	40.74	68.38	32.82	1.105
#19	KS	29	F		200	2.008	1.138	6.086	1.976	12.96	1.074
#20	SP	30	F		190	4.240	0.703	45.02	82.47	7.216	0.406
#21	NC	42	M	+	350	3.223	0.479	32.45	56.08	89.02	0.332
#22	SR	24	F		360	5.626	1.072	21.73	39.20	15.73	ND
#23	WR	60	M	+	500	12.7	1.25	23	56.48	32.93	1.26
#24	PMc	38	F		950	21.29	0.304	19.30	14.08	53.46	2.983
#25	DB	39	F		300	3.603	0.657	13.92	22.34	14.30	0.702
#26	GW	70	F		250	3.38	0.295	4.42	14.67	3.477	0.29
#27	PS	21	M		250	3.187	0.36	13.74	24.94	20.47	0.407
#28	RR	46	M		810	5.799	0.121	4.787	8.602	6.326	0.356
#29	MB	25	M		440	9.521	1.020	11.19	24.93	16.19	1.161
#30	AK	69	M		500	7.91	0.445	15.1	29.93	21.37	0.365
#31	MC	24	F		300	4.239	1.032	18.06	53.43	73.40	ND
#32	RD	29	M		530	10.57	0.588	24.41	60.51	66.49	0.810
#33	MT	43	M		650	9.035	0.442	24.90	47.83	28.04	1.033

#34	FS	43	F		600	9.69	0.582	25.99	47.18	72.72	1.506
#35	JT	19	M		310	8.884	0.849	12.41	27.39	14.10	0.570
#36	MT	39	M		300	3.141	0.9	10.16	21.04	6.828	0.585
#37	DT	21	M		150	1.876	0.297	15.00	27.76	16.84	0.432

Appendix 8b -Urinary caffeine metabolites in relatives of patients with FAP.

Vol		Age	Sex	Smoker	Urine vol (ml)	Amount excreted in urine (mg)					
						17-DMX	17-DMU	1-MX	1-MU	AAMU	CAFF
#1	AL	26	M	+	390	4.083	0.273	13.65	33.99	26.70	0.347
#2	DC	46	M	+	600	9.582	0.432	12.48	44.04	19.10	0.33
#3	TK	20	F	+	220	4.072	0.475	25.39	54.35	81.98	0.33
#4	ML	65	M		610	10.65	1.006	9.686	29.09	60.98	1.335
#5	GL	18	F	+	650	6.545	1.014	30.97	48.00	48.63	1.124
#6	KP	23	F	+	900	9.189	0.36	16.28	34.2	46.50	0.828
#7	MH	27	M		250	2.597	0.487	10.79	25.33	12.00	0.542
#8	MT	22	M		450	8.959	0.747	21.66	41.04	37.06	0.949
#9	AF	20	F		850	15.83	1.555	40.38	56.85	39.61	2.38
#10	HS	53	F	+	650	9.737	0.773	24.02	56.93	36.22	1.859
#11	SC	15	M		225	2.938	0.465	23.29	36.35	31.49	0.247
#12	TT	18	F		220	2.171	0.239	5.599	7.893	9.385	0.338
#13	RC	24	M		350	8.522	0.626	29.12	58.45	76.50	0.577
#14	MP	53	F		650	13.96	0.364	6.63	11.30	41.17	4.049
#15	SP	19	M		300	1.707	0.048	1.887	3.456	3.489	0.255
#16	GG	51	M		325	2.083	0.159	2.827	7.683	10.47	0.536

Appendix 9.

Recovery and measurement precision of nonpolar caffeine metabolites in 95:5% chloroform: isopropanol.

	Caffeine	1,7-DMX	*DMMIOD	1,3-DMX	3,7-DMX
Extraction Efficiency (%)	99.8	95.5	82	99	92
CV % (n= 5)	4.0	2.8	8.4	2.2	9.1

* Originally thought to be the caffeine metabolite 1,7-DMU.

Recovery and measurement precision of polar caffeine metabolites in chloroform: isopropanol.

	3-MU	7-MU	1-MU	3-MX	7-MX	1-MX	1,3-DMU	3,7-DMU
95:5% v/v								
Extraction Efficiency	3.0	2.8	3.2	21		33	22	27
CV % (n=5)	0.1	2.9	4.0	5.9		1.8	3.0	1.4
50:50% v/v								
Extraction Efficiency	68.7	74.6	83.7	79.7	75.5	107	106.4	101
CV % (n=5)	4.2	5.7	5.4	1.4	8.7	6.7	7.5	2.9

Appendix 10.

The stability of caffeine and its metabolites at -22°C

Months @ -22°C	TMX	1,7-DMX	1,7-DMU	1-MX	1-MU	AAMU
			(ug/ml)			
t0	4.84	13.12	1.03	45.56	50.71	73.32
t1	4.86	12.90	1.03	47.04	52.21	72.83
t2	4.75	13.13	1.04	46.73	54.88	74.12
t3	4.80	13.39	1.02	47.02	53.29	71.08
% Change	0.83%	2.06%	0.99%	3.20%	5.09%	3.15%

The stability of caffeine and its metabolites at 24°C

Hours @ 24°C	TMX	1,7-DMX	1,7-DMU	1-MX	1-MU	AAMU
			(ug/ml)			
t0	4.84	13.12	1.03	45.56	50.71	73.32
t8	5.02	13.20	1.04	46.22	53.38	73.44
t16	4.86	13.14	1.02	46.46	52.79	71.89
t24	4.90	13.15	1.03	46.35	52.81	73.03
% Change	1.24%	0.23%	0%	1.73%	4.14%	0.4%

Appendix 10 contd.

The stability of caffeine and its metabolites at 37°C

Hours @ 37°C	TMX	1,7-DMX	1,7-DMU	1-MX	1-MU	AAMU
			(ug/ml)			
t0	4.84	13.12	1.03	45.56	50.71	73.32
t2	4.80	13.10	1.04	45.81	52.64	72.89
t4	4.93	13.28	1.00	45.23	52.39	72.89
t10	4.78	13.30	1.06	46.57	52.41	72.97
% Change	1.26%	1.37%	2.91%	2.22%	3.35%	0.48%